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SECTOR 4

0120

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant(s):	)	
	)	
Jeffrey Kenneth Horton	)	Title: In-Situ Extraction and
	)	Assay Method
Serial No: 09/027,654	)	
	)	
Filed: February 23, 1998	)	

TRANSMITTAL OF EXECUTED DECLARATION

Assistant Commissioner for Patents
Washington, D.C. 20231

Attention: BOX MISSING PART

Sir:

Submitted herewith is an executed Declaration for filing in the above-identified application, in response to the Notice to File Missing Parts issued by the Patent and Trademark Office on May 12, 1998.

06/29/1998 MPEOPLES 00000036 09027654
01 FC:105 130.00 OP

CERTIFICATE OF MAILING (37 CFR 1.8)

I hereby certify that this paper and the documents referred to as enclosed therewith are being deposited with the United States Postal Service as first class mail, postage prepaid, on June 16, 1998 in an envelope addressed to Assistant Commissioner for Patents, Washington, D.C. 20231.

Jeffrey S. Sharp

Also enclosed is a copy of the Notice together with our check in the amount of $130.00 in payment of the fee.

The Commissioner is hereby authorized to charge any deficiency in the amount enclosed or any additional fees which may be required under 37 CFR 1.16 or 1.17 to Deposit Account No. 13-2855. A copy of this request is enclosed.

Please refund any overpayment to Marshall, O'Toole, Gerstein, Murray & Borun at the address below.

Respectfully submitted,

MARSHALL, O'TOOLE, GERSTEIN,
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By: [signature]
Jeffrey S. Sharp
Reg. No: 31,879

June 16, 1998

PATENT APPLICATION

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Docket No: 28911/34561

PATENT APPLICATION TRANSMITTAL UNDER 37 C.F.R. 1.53

Box Patent Application
Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

Transmitted herewith for filing is the patent application of

Inventor(s): Jeffrey Kenneth Horton

Title: *In-Situ* Cell Extraction and Assay Method

1. Type of Application

☒ This is a new application for a
- ☒ utility patent.
- ☐ design patent.

☐ This is a continuation-in-part application of prior application no.

2. Application Papers Enclosed

1	Title Page
57	Pages of Specification (excluding Claims, Abstract & Drawings)
2	Page(s) of Claims
1	Page(s) of Abstract
5	Sheet(s) of Drawings (Figs. 1 to 10)

☒ Formal

☐ Informal

CERTIFICATION UNDER 37 CFR 1.10

I hereby certify that this Patent Application Transmittal and the documents referred to as enclosed therewith are being deposited with the United States Postal Service on **February 23, 1998**, in an envelope addressed to the Assistant Commissioner for Patents, Washington, D.C. 20231 utilizing the "Express Mail Post Office to Addressee" service of the United States Postal Service under Mailing Label No. EM014372758US.

Richard Zimmermann

3. **Declaration or Oath**

- ☐ Enclosed
  - ☐ Executed by (check all applicable boxes)
    - ☐ Inventor(s)
    - ☐ Legal representative of inventor(s) (37 CFR 1.42 or 1.43)
    - ☐ Joint inventor or person showing a proprietary interest on behalf of inventor who refused to sign or cannot be reached
      - ☐ The petition required by 37 CFR 1.47 and the statement required by 37 CFR 1.47 are enclosed. See Item 5D below for fee.

- ☒ Not enclosed - the undersigned attorney or agent is authorized to file this application on behalf of the applicant(s). An executed declaration will follow.

4. **Additional Papers Enclosed**

- ☐ Preliminary Amendment
- ☐ Information Disclosure Statement
- ☐ Declaration of Biological Deposit
- ☐ Computer readable copy of sequence listing containing nucleotide and/or amino acid sequence
- ☐ Microfiche computer program
- ☐ Verified statement(s) claiming small entity status under 37 CFR 1.9 and 1.27
- ☐ Associate Power of Attorney
- ☐ Verified translation of a non-English patent application
- ☐ An assignment of the invention
- ☒ Return receipt postcard
- ☐ Other

5. **Priority Applications Under 35 USC 119**

Certified copies of applications from which priority under 35 USC 119 is claimed are listed below and

☐ are attached.

☒ will follow.

COUNTRY	APPLICATION NO.	FILED
EPO	97301398.0	March 3, 1997
Great Britain	9715704.4	July 24, 1997

6. **Filing Fee Calculation (37 CFR 1.16)**

A. ☒ **Utility Application**

CLAIMS AS FILED - INCLUDING PRELIMINARY AMENDMENT (IF ANY)						
			SMALL ENTITY		OTHER THAN A SMALL ENTITY	
	NO. FILED	NO. EXTRA	RATE	FEE	RATE	FEE
BASIC FEE				$395.00		$790.00
TOTAL	14 -20	= 0	X 11 =	$	X 22 =	$0.00
INDEP.	2 - 3	= 0	X 41 =	$	X 82 =	$0.00
☐ First Presentation of Multiple Dependent Claim			+ 135 =	$	+ 270 =	
Filing Fee:				$	**OR**	$790.00

B. ☐ **Design Application ($165.00/$330.00)** Filing Fee: $ _____

C. ☐ **Plant Application ($270.00/$540.00)** Filing Fee: $ _____

D. **Other Fees**

☐ Recording Assignment [Fee -- **$40.00** per assignment] $ _____

☐ Petition fee for filing by other than all the inventors or person on behalf of the inventor where inventor refused to sign or cannot be reached [Fee -- **$130.00**] $ _____

☐ Other $ _____

Total Fees Enclosed $790.00

7. **Method of Payment of Fees**

☒ Enclosed check in the amount of: $790.00

☐ Charge Deposit Account No. 13-2855 in the amount of: $______
A copy of this Transmittal is enclosed.

☐ Not enclosed

8. **Deposit Account and Refund Authorization**

The Commissioner is hereby authorized to charge any deficiency in the amount enclosed or any additional fees which may be required during the pendency of this application under 37 CFR 1.16 or 37 CFR 1.17 or under other applicable rules (except payment of issue fees), to Deposit Account No. 13-2855. A copy of this Transmittal is enclosed.

Please refund any overpayment to Marshall, O'Toole, Gerstein, Murray & Borun at the address below.

Please direct all future communications to Jeffrey S. Sharp, at the address below.

Respectfully submitted,

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By: ______________________
Jeffrey S. Sharp
Reg. No: 31,879

February 23, 1998

SOLE INVENTOR

"EXPRESS MAIL" mailing label No. EM014372758US.
Date of Deposit: February 23, 1998
I hereby certify that this paper (or fee) is being deposited with the United States Postal Service "EXPRESS MAIL POST OFFICE TO ADDRESSEE" service under 37 CFR §1.10 on the date indicated above and is addressed to: Assistant Commissioner for Patents, Washington, D.C. 20231

Richard Zimmermann

APPLICATION FOR UNITED STATES LETTERS PATENT

SPECIFICATION

TO ALL WHOM IT MAY CONCERN:

Be it known that I, Jeffrey Kenneth Horton, a citizen of Britain, residing at 11 Pwll-Y-Myn Crescent, Wyndham Park, Peterston-Super-Ely, Cardiff, CF5 6LR, United Kingdom, have invented a new and useful *In-Situ* Cell Extraction and Assay Method, of which the following is a specification.

IN-SITU CELL EXTRACTION AND ASSAY METHOD

FIELD OF THE INVENTION

The present invention relates to the field of immunoassays. The invention provides a simple and convenient, single stage, single vessel cell extraction and assay method which is suitable for the extraction and measurement of a range of different types of analyte which occur as cellular components. The invention also relates to kits of reagents suitable for performing cellular extraction and measurement as a single stage, single vessel process.

BACKGROUND TO THE INVENTION

1. Immunoassay Technology

A number of techniques are known and have been described for the measurement of small quantities of biological materials. Of these techniques, the area of immunoassays has been extensively reviewed and the technique forms the basis of many commercially available assay kits.

For more than thirty years, immunoassay has been the method of choice for measuring low analyte concentrations in complex biological fluids. The procedure is equally applicable to the measurement of low molecular weight compounds such as drugs, steroids and the like, as well as large molecular weight compounds such as protein molecules. The technique combines sensitivity and specificity. Immunoassays are used in basic biological research to investigate the physiological and possible pathological role of a wide range of potent biologically active substances, including cyclic nucleotides, prostglandins, leukotrienes, growth factors, steroid hormones and cytokines. Such research often leads to the identification of new therapeutic agents. Immunoassays are

often used in the pharmaceutical industry in many aspects of drug development processes. These range from drug screening, toxicological, pharmacological and pharmacokinetic studies, through to clinical trials. Immunoassays have had their greatest impact in the area of clinical diagnostic tests. The technique has been employed for many years in hospital clinical biochemistry laboratories to diagnose disease and metabolic disorders. The technique was introduced in 1959 by Berson & Yalow. (Yalow, R.S. and Berson S.A., Assay of plasma insulin in human subjects by immunologic methods, Nature, (1959), 184, 1684). The combination of a signal which could be detected and a protein molecule (an antibody) which binds specifically and avidly to the analyte of interest lies at the heart of all immunoassay procedures. Assay designs have proliferated over the last thirty years, as have the different types of signal reagents and detection systems. Sophisticated instruments with associated computer hardware have been developed with the aim of increasing sample throughput. Further background information relating to immunoassay techniques can be found in 'The Immunoassay Handbook, (Wild, D.G. Ed, Stockton Press, New York, (1994), which deals with many of the concepts associated with immunoassay technology which are pertinent to the present invention. It considers, for example, competitive (also termed 'labelled analyte' or 'limited reagent') and immunometric ('labelled antibody' or 'reagent excess') systems.

The earliest methods were those which involved a step of separating the bound analyte from the free, in order to be able to measure the amount of bound analyte. Various separation methods have been described, including charcoal absorption, ammonium sulphate precipitation, magnetic particles ('Amerlex™'), etc. More recently, solid supports have been utilised for immunoassay procedures, including the walls of microtitre well plates.

A more recent development has been the introduction of

homogeneous radioimmunoassay technology, notably the technique of scintillation proximity assays (SPA) covered by US Patent No. 4,568,649. Scintillation proximity assay is a radioisotopic assay technique which has gained wide acceptance in recent years, and is applicable to radioimmunoassays, as well as to radio-receptor and enzyme assays. The technique relies on the observation that β-particles emitted from radioisotopes will travel only a limited distance in an aqueous environment (in the case of tritium β-particles, this is 1.5μm), before the energy is dissipated. In SPA, the target of interest is immobilised to a small microsphere containing scintillant. When a radioisotopically labelled molecule is brought into close proximity with the microsphere, β-particle energy is transferred effectively to the scintillant, thereby causing the emission of light. Labelled molecules which remain free in solution are undetected because they are too distant from the scintillant-containing microsphere. In a typical radioimmunoassay, the microsphere is coated with a capture moiety, such as protein, A, or secondary antibodies, such as donkey-anti rabbit, sheep-anti-mouse antibodies. A sample, containing or suspected of containing the analyte (i.e. antigen) to be tested, is incubated in the presence of an antibody specific for that analyte, together with a quantity of a radiolabelled analyte. The antibody/analyte complex is captured by the secondary antibody and is detected by the emission of light. Any labelled antigen which remains unbound by the antibody, will be free in solution and be undetected. The assay therefore requires no separation step and the protocol has fewer pipetting steps compared with conventional, i.e. separation-based radioimmunoassays. It has been shown that in SPA-based assays, there is often an increase in assay precision and reproducibility, compared with traditional separation-based assays. Another advantage lies in the potential for increased sample throughput and capability for automation. (Cook, N.D., Drug Discovery Today (1996), 1, 287-294). The application of SPA to RIA methodology is

not restricted to particular analytes or to types of molecule and in principle the technique can be applied in place of traditional separation-based assays. Some examples of RIAs developed using SPA are shown in Table 1.

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Table 1. Examples of Radioimmunoassays Developed using SPA

Assay	Reference
Cyclic AMP	Horton JK & Baxendale PM (1995), In: Methods in Molecular Biology,41,pp. 91-105, Eds. Kendall, DA and Hill, SJ, Humana Press Inc, Towota, NJ
Cyclic GMP	Heath R Bryant B & Horton JK (1992), In: The Biology of Nitric Oxide. Part 2. Enzymology, Biochemistry and Immunology pp. 98-102, Eds. Moncada , S *et al.* Portland Press
6-Keto-Prostoglandin F1 alpha	Baxendale PM *et al* (1990) In: Advances in Prostaglandins, Thromboxane and Leukotriene Research, 21, pp. 303-306, Eds. Samuelsson, B. *et al*, Raven Press
Acyclovir	Tadepalli, SM, Topham, PA & Quinn, RP. (1990) Clin. Chem. 36, 1104
Platelet Activating Factor	Sugatani , J *et al* (1990), Life Sciences, 46,1443-1450
Abscisic Acid	Whitford, PN. & Croker, SJ. (1991) Phytochemical Analysis, 2, 134-136
Androstenedione	Fiet, J. *et al* (1991) Clin. Chem., 37,293
Ranitidine	Linacre, P. & Morris, SE, (1992), In: Bioanalytical Approaches for Drugs, including anti-asthmatics and metabolites, 22, pp.325-326 Eds. Reid, E. & Wilson, ID. Royal Society of Chemistry, London

More recently, alternatives to scintillant-containing beads (fluomicrospheres) have been described for use in proximity assays. PCT Application No. WO 90/03844 (Wallac) discloses a microtitre well plate intended for binding assays. The sample plate is produced from a transparent scintillant-containing plastic by means of a vacuum thermoforming or injection moulding process. In principle, the walls of the microtitre well plate can be coated with a binding compound for the purpose of performing *in vitro* binding assays using radiolabelled reactants.

PCT Application No. WO 94/26413 discloses an apparatus and a method for studying cellular biochemical processes in real time. In one aspect, the application describes a multiwell plate, such as a microtitre well plate, in which the base of the plate is formed from a scintillant plastic material and the walls are formed from an opaque plastic material, the wells of the plate being adapted for the attachment or growth of cells. The scintillating microplates are designed for use in the real-time analysis of a wide spectrum of cell associated phenomena, and applications have been demonstrated in transport, cell motility, uptake, metabolism and other cell based processes. Cytostar™ scintillating microplates form the basis of a new technology introduced by Amersham International plc, for the study of cellular processes. In other applications, the scintillating microplates can be used for *in vitro* assays, for the measurement of ligands, analytes, etc. In this format a binding compound is bound to the walls of the microtitre well plate for reaction with label and analyte.

As an alternative to radioisotopic methods for performing immunoassays, non-radioactive systems have been introduced. Today, enzymes are the most widely used tracers. When in combination with colourimetric end-points, they provide highly sensitive, robust, precise, accurate and convenient immunoassays. A major breakthrough came with the introduction of ninety-six well microtitre plates. Inexpensive automatic colourimetric multiwell plate readers are available. A number of other non-

isotopic labels have been described, of which luminescent and fluorescent labels are the most popular.

2. Cell Extraction Methods

Traditional methods for immunoassay depend on obtaining the samples in a sufficiently suitable state, i.e. sufficiently free from interfering factors . Usually this will involve a cellular extraction method. Numerous procedures are described which detail the extraction of intracellular molecules from cells. Typically these methods involve acid, solvent or solid phase methods to accomplish cell lysis and extraction of the molecule of interest. Methods for performing such extractions can be found in several publications. Further background information relating to cell extraction methods can be found in a review article by Goldberg & O' Toole. (Goldberg, ND & O'Toole, AG (1971); In: Methods of Biochemical Analysis, 20, Ed Glick D. pp 1-39 Interscience Publishers, Wiley, London)

Examples of cellular extraction methods are as follows.

2.1 Solvent Extraction (Horton & Baxendale, 1995; See Table 1 for Reference)

Ice-cold ethanol is added to cell cultures to give a final suspension volume of 65% (v/v) ethanol and the suspension allowed to settle. The supernatant is aspirated into test tubes, the remaining precipitate washed with ice-cold 65% (v/v) ethanol and the washings added to the appropriate tubes. The extracts are centrifuged at 2000g for 15 minutes at 4°C and the supernatant transferred to fresh tubes. The combined extract is then dried overnight, either under a stream of nitrogen at 60°C, in a vacuum oven for 8 hours, or in a centrifugal evaporator on a high temperature setting for 4 hours.

In this procedure, there is a possibility of overdrying, and this can result in difficulty in reconstituting the samples. The dried extracts are

dissolved in a suitable volume of assay buffer before analysis.

2.2 Acid Extraction

Hancock *et al*, (J. of Receptor & Signal Transduction Research, 1995, 15, 557-579) describe an acid extraction method for intracellular molecules in which 0.2M hydrochloric acid is added to cells, and each separate sample is vortex mixed for 1-2 minutes. The sample is carefully neutralised to a pH that is compatible to the immunoassay, using stepwise addition of 10µl aliquots of 2.5M sodium hydroxide, care being taken to measure the pH of the sample after each addition of alkali. This step is particularly critical, as the use of a non-optimal pH with an immunoassay can result in inaccurate measurement or non-measurement of analyte in the samples.

An alternative approach is described by Steiner, (In: Methods of Hormone Radioimmunoassay ,1979, Eds Jaffre, BM, & Behrman, HR pp. 3-17 Academic Press, New York), in which cell samples are homogenized in cold 6% (w/v) trichloroacetic acid at 4°C to give a 10% (w/v) slurry. The sample is centrifuged at 2000g for 15 minutes at 4°C. The supernatant is reserved and the pellet discarded. The supernatant is washed four times with five volumes of water-saturated diethyl ether, discarding the upper layer after each wash. The aqueous extract is lyophilized overnight or dried under a stream of nitrogen at 60°C overnight and the dried extract dissolved in a suitable volume of assay buffer before analysis.

2.3 Solid Supports (e.g. Ion Exchange or 'Amprep' columns)

A protocol for the extraction of intracellular molecules by ion exchange chromatography, using disposable minicolumns, has been described previously (Horton & Baxendale, 1995; see Table 1 for Reference). The columns (for example, ion exchange SAX columns) are

used with a vacuum manifold and a vacuum pump. The columns are prepared by applying a vacuum and rinsing with 2ml 100% methanol, followed by washing with 2ml of water, taking careful precautions so as not to allow the solid support to dry, or to allow the flow rate to exceed 5ml/minute. The cultured cells are applied directly to the column and washed with 3ml 100% methanol. Three millilitres of acidified methanol (prepared by diluting concentrated hydrochloric acid to 0.1M with absolute methanol) is added to the column and the eluate collected. The fractions are dried using a stream of nitrogen or in a vacuum oven overnight (see above). The samples are reconstituted in assay buffer, as described above, before assay.

2.4 Detergent methods

Other methods are known for the extraction of nucleic acid samples and nucleotides such as ATP. For example, Lundin and Anson (PCT WO 92/12253) describe a method for extracting an intracellular component in which bacterial cells are lysed with a detergent which is subsequently neutralised by addition of a cyclodextrin. A cellular component (e.g. ATP, DNA or RNA) liberated is subsequently measured or processed using biochemical or molecular biology (non-immunoassay) techniques such as the firefly luciferase and polymerase chain reaction assays. No reference is made in this patent application to one-step assays, homogeneous immunoassays, including scintillation proximity assay methods, or separation based immunoassay techniques.

EP 0 309 184 (Lumac) describes a method for the extraction of ATP from a microorganism with an ATP releasing agent and contacting the resultant solution with a neutralising agent which acts substantially to eliminate the distorting effect the releasing agent on the subsequent ATP assay. In EP 0 309 184 the releasing agent is preferably a cationic surface active agent which is preferably contacted with a non-ionic surface active

neutralising agent.

The use of cyclodextrins to remove surfactants from solutions and surfaces and surfaces has been described previously in European Patent Application EP 301 847 (P. Khanna and R. Dworschack). According to this patent application, surfactants can be removed and cleaned from solutions and containers used in biochemical reactions by immobilised cyclodextrins. EP 286367 (Khanna *et al*) describes cyclodextrins as neutralisers of surfactants used as storage stabilisers for enzymes which are used as tracers in enzyme immunoassays. In a review, various applications of cyclodextrins in biological and chemical reactions have been described. (J. Szejtli, Cyclodextrins in Diagnostics, Kontakte [Darmstadt] 1988 [1]. 31-36).

The use of cyclodextrins, to neutralise surfactants added as extractants to release intracellular molecules, in a simple, single-step extraction and measurement immunoassay system has not been described previously.

All of the above prior art methods for immunoassays suffer from a number of disadvantages, including:

i) Unable to process large numbers of cells samples

ii) Time consuming

iii) Labour intensive

iv) Prone to errors because of the large number of steps

v) The need to remove the cell extraction reagent before further processing and measuring can take place. If this is not carried out, then accurate measurements may not take place, or, indeed, could result in total assay inhibition, and therefore measurement of the substance in the cellular extract is prevented.

In all of the traditional methods of sample preparation for radioimmunoassay, it has hitherto been necessary to perform separate lysis and extraction processes in order to obtain samples in a suitable form

for subsequent measurement. The prior art methods therefore involve three separate processes which must be carried out sequentially, thereby adding to the time and cost of each immunochemical assay. In addition none of the prior art methods for the assay of intracellular components would be amenable to high throughput screening methods which are necessary if large numbers of samples are required to be processed. In this specification, data is presented whereby addition of a cellular lysis reagent to an immunoassay system, results in inhibition of antigen:antibody binding; and the inclusion of complex carbohydrates, such as cyclodextrins, restores the antigen:antibody binding event. In the preferred embodiment of the invention, 1% DTAB (dodecyl trimethyl ammonium bromide) is employed as a cellular lysis reagent (which inhibits antigen:antibody binding) and 2.5% alpha cyclodextrin is used as a sequestration reagent restoring antigen:antibody binding. These reagents, together with homogeneous immunoassay techniques, have enabled, for the first time, the establishment of a concerted one stage, single pot, cellular lysis and immunoassay system for the accurate measurement of intracellular molecules.

Thus, a novel, convenient and rapid method for the extraction and quantitation of target molecules is described here, which permits the growth of cells, the extraction of intracellular components and the subsequent assay of such components to be carried out in the same vessel. The technique is simple to perform and can be carried out with little technical intervention. Since few manipulations are necessary, the procedure is fully amenable to robotic automation

DESCRIPTION OF THE INVENTION

The invention provides a method of assaying for an analyte which method comprises the steps of:

i) mixing a sample of cells possibly containing the analyte with

a cell lysis reagent to provide a cell lysis fluid,

ii) mixing the cell lysis fluid with reagents, including a specific binding partner of the analyte for binding to the analyte, for performing a specific binding assay for the analyte,

iii) and mixing the cell lysis fluid with a sequestrant for the cell lysis reagent, whereby the binding of step ii) is performed in the presence of the sequestrant.

The analyte is a cellular component. Any cellular component for which a specific binding partner is available can in principle be utilised in the invention. Typical specific-binding partner combinations suitable for use with the invention may be selected from: hapten-antibody, ligand-receptor, DNA-DNA, RNA-RNA, DNA-RNA, biotin-streptavidin, protein-antibody, peptide-antibody, and polypeptide-antibody interactions. Preferably the specific binding assay is a protein-binding assay or particularly an immunoassay. Preferred cellular components include proteins, peptides, second messengers such as cyclic AMP and cyclic GMP, hormones, steroids, peptides, prostaglandins, inositol phosphates, cytokines chemokines and leukotrienes.

The assay may be designed to measure an analyte present within the cells, in which case the cells will usually be separated from a cell culture medium prior to lysis. Or the assay may be designed to measure an analyte present in both intracellular and extracellular fluids, in which case the cells will usually be lysed in the presence of a medium in which they have been cultured.

The cell lysis reagent is preferably a detergent, that is to say a surface active agent which may be cationic, anionic, zwitterionic or non-ionic. Examples of suitable detergents include dodecyl trimethyl ammonium bromide (DTAB); cetyl pyridinium chloride (CPC); benzethonium chloride (BZC); sodium dodecyl sulphate (SDS), and N-dodecyl-N,N-dimethyl-3-ammonio-1-propane sulphonate (DDAPS). DTAB,

CPC and BZC are cationic surfactants; DDAPS is a zwitterionic surfactant and SDS is an anionic surfactant. The use of these detergents as cell lysis agents is well known in the field. Typical concentrations are in the range of 0.4 - 4% by weight on the weight of the cell lysis fluid. If too little detergent is used, then cell lysis may be slow or incomplete. In addition to lysing cells in order to release an intracellular component into a cell lysis fluid, the detergent may also adversely affect the binding of that intracellular component to its specific binding partner added in the course of step ii) for assay. The sequestrant is used to inhibit or annul that undesired adverse effect.

A key feature of the invention is the use of a sequestrant for the cell lysis reagent. The sequestrant acts to prevent the cell lysis reagent from adversely affecting a binding reaction between the analyte and its specific binding partner. The sequestrant may do this e.g. by chemically reacting with the cell lysis reagent or by physically absorbing it. Preferred sequestrants are carbohydrates such as cyclodextrins. Cyclodextrins are toroidal molecules consisting of 6, 7 or 8 glucose units (α-, β- and γ-cyclodextrin). The interior of the ring binds a hydrophobic tail of a molecule such as a surfactant. The resultant inclusion complex is generally formed with a 1:1 stoichiometry between surfactant and cyclodextrin. γ-Cyclodextrin and particularly α-cyclodextrin are preferred for use in this invention. Preferably enough sequestrant is used to be capable of sequestering or inactivating all the cell lysis reagent present. Preferably the amount of sequestrant is from 0.5 - 10%, particularly 1 - 5%, by weight on the weight of the reaction mixture.

Clearly, the method described herein can be readily adapted for use with traditional separation, non-homogeneous immunoassays, and also two-stage methods whereby cells are cultured in separate vessels from those used to carry out the immunoassay measurements.

It is an advantage of the invention that steps i), ii) and iii) can

all preferably be performed in a single reaction vessel. The cells from which the analyte is extracted in step i) may be dead but are preferably living. It may be convenient to culture the cells in the reaction vessel in which the assay is to be performed. Preferably multiple assays are performed in parallel in wells of a multiwell plate such as a microtitre plate. If desired, the contents of individual wells of a multiwell plate can be transferred to individual wells of another multiwell plate at any stage during performance of the method.

Preferably the cell lysis fluid that results from step i) is used, without any intermediate separation or purification, for performing steps ii) and iii). Preferably the sequestrant is included in one of the reagents that is mixed with the cell lysis fluid in step ii). Thus the components present in an assay according to the invention may typically comprise:

a) a source of cells possibly, or suspected of, containing the analyte;

b) a cell lysis reagent;

c) an unlabelled specific binding partner of the analyte which is, or is capable of being, immobilised on a solid support;

d) a specific binding partner, or an analogue, of the analyte, which is either labelled or unlabelled and capable of being labelled.

One or both of components c) and d) includes a sequestrant, the order of addition of components c) and d) being immaterial.

In one format of the invention, the immunoassay is a scintillation proximity assay. In this format, components a), b), c) and d) are contained in the wells of a microtitre well plate, component d) being a radioactively labelled analogue of the compound being tested for. The scintillation proximity assay measurement is initiated by the addition to the wells of SPA fluomicrospheres coated with a binding reagent such as a secondary antibody or protein A.

Alternatively, cells may be cultured in the wells of a scintillant

microtitre well plate suitable for the purpose, the base and/or walls of the wells being coated with a binding reagent such as a secondary antibody or protein A. After a suitable time, the remaining assay components, b), c) and d) are added to the wells.

In a second format of the invention, the immunoassay is an enzyme-immunoassay. In this format, components a), b), c) and d) are contained in the wells of a microtitre well plate, component d) being an enzyme-labelled specific binding partner of the compound being tested for. The assay measurement is initiated by the addition to the wells of detection reagents suitable for the detection of the enzyme label.

Suitable sequestration reagents are chosen from the group consisting of complex carbohydrates, including cyclodextrins. In a preferred format, alpha-cyclodextrin is employed in the method of this invention.

The method comprises incubating cells with cell lysis buffer, adding to the mixture of lysed cells, the labelled specific binding partner for the substance, followed by addition of the unlabelled specific binding partner, both reagents being dissolved in a buffer containing sequestration agent, and measuring the signal generated by the labelled specific binding partner as a measure of the amount of substance or component, with the entire quantities of reagents present in the reaction vessel at the same time. The signal obtained may be compared with the signals obtained using a set of standard quantities of substance using a parallel procedure and generating a standard curve for the assay.

In another aspect the invention provides a kit, for assaying for an analyte by the method described, comprising: a detergent; a sequestrant for the detergent; a specific binding partner of the analyte; a tracer; and separation means for separating bound tracer from unbound tracer. The tracer is a labelled assay reagent, which might be the specific binding partner of the analyte or might be another assay reagent. Separation means envisaged include assay reagents which are

immobilised e.g. on SPA beads or magnetic beads or on an inner surface of the assay vessel. The kit may also include an analyte standard and a buffer.

In one format, the immunoassay process is a radioimmunoassay, in which the labelled specific binding partner contains a radioisotope. Suitable radioisotopes for use in the assay method of the present invention include β-emitting isotopes such as tritium, and iodine-125, which emits Auger electrons.

In an alternative format, the immunoassay process is an enzyme-immunoassay, in which the labelled specific binding partner is, or can be, bound to an enzyme label. Typical enzyme labels suitable for use in the present invention are alkaline phosphatase, β-galactosidase, horseradish peroxidase, malate dehydrogenase and glucose-6-phosphate dehydrogenase. Horseradish peroxidase is a particularly preferred enzyme label for use in the enzyme immunoassay method according to the present invention.

In another format, the labelled specific binding partner can include a fluorescence label. Suitable fluorescent labels for use in the present invention may be selected from fluorescein, rhodamine and cyanine dyes.

The precise assay format, choice of specific binding partner, the detection label, and the nature of the substance to be tested for are not critical to the present invention. Rather, the invention relies on the unexpected observation that a precise measurement of intracellular components can be made without the separate extraction and/or purification procedures being performed on the cell samples which are inherent in, and characterise the prior art methods.

Illustrative of the immunoassay methods which can be utilised in the present invention are the following assay formats.

I) **Radioactive Assays**

Scintillation Proximity Assay using Scintillant Beads

The method provides a simple, single-step lysis and measuring method for intracellular components. The immunoassay reagents (antisera, tracer, SPA beads) are added to the same wells which are used for growing cells. The process is carried out in single wells without further technical intervention. In this aspect of the invention there is a requirement for cultured cells grown in a suitable vessel. In a preferred form of the invention there is a requirement for a tissue-culture treated microtitre plate with opaque walls and a clear base, to allow microscopic inspection of the cells. A lysis reagent is added to the cultured cells, followed by labelled specific binding partner, unlabelled specific binding partner and second antibody derivatised scintillant beads prepared in buffer containing the sequestration agent. Standards are added to empty microtitre wells on the same plate. The plate is incubated for a suitable time period before counting on a β-scintillation counter. The concentration of analyte in the samples is determined by interpolation from a standard curve.

Alternatively, following the lysis event, a specific binding partner coupled to scintillant beads is incubated with the antigen, together with a second specific binding partner. The second binding partner is unlabelled and detection is through a third binding reagent which is labelled.

Alternatively, following the lysis event, the antigen/second specific binding partner complex is bound to the scintillant beads, the second specific binding partner being unlabelled and detection is through a third binding reagent which is labelled.

Scintillation Proximity Assays using Scintillating Microtitre Plates

In an alternative for of the assay system described above, a

Cytostar-T plate (or equivalent) is used. In a preferred embodiment of the invention, there is a requirement for a sterile, tissue-culture-treated scintillant microtitre plate with opaque walls and a clear base to allow microscopic inspection of the cells. In this method, the plate is pre-coated with specific or secondary antibodies. As above, this method provides a simple, single-step lysis and measuring method for intracellular components of cultured cells grown in the Cytostar-T plate (or equivalent). The immunoassay reagents (tracer, +/- antisera) are added to the same wells which are used for growing cells. A lysis reagent is added to the cultured cells, followed by tracer (or antisera depending on whether primary or secondary antibodies have been used to coat the plate) dissolved in buffer containing the sequestration reagent. Standards are added to empty wells on the same plate. The plate is incubated for a suitable time period before counting on a β-scintillation counter. The concentration of analyte in the sample is determined by interpolation from a standard curve.

II **Enzyme Immunoassays**

'EMIT' type (Rubenstein KE. *et al*, 1972. Biochem. Biophys. Res. Comm. 47; 846)

The enzymes malate dehydrogenase and glucose-6-phosphate dehydrogenase have been used extensively in the homogeneous immunoassay exemplified by the EMIT (Enzyme Multiplied Immunoassay Technique) system. Both enzymes are monitored by the conversion of the cofactor NAD to $NADH_2$ in a spectrophotometer at 340nm. In this assay system, the analyte competes with labelled antigen for antibody binding sites. The activity of the enzyme is modified when the antibody binds to the labelled antigen.

Cells are cultured in a sterile, clear tissue culture treated microtitre plate, lysed and then the other components of the homogeneous

EMIT EIA are added dissolved in buffer containing the sequestration (cyclodextrin) agent. Optical density is measured and the concentration of analyte in the samples is determined by interpolation from a standard curve. Standards are added to empty wells of the same plate that are not used for growing cells.

'CEDIA' type (Henderson DR *et al* 1986 Clin. Chem.32,1637-1641)

In the cloned enzyme donor immunoassay method, two inactive fragments of β-galactosidase have been synthesized by genetic engineering. The large fragment, the enzyme acceptor, contains 95% of the enzyme, and the small fragment, the enzyme donor, consists of the remaining 5%. On mixing, the two fragments aggregate into tetramers which have β-galactosidase enzyme activity. In this assay, antigen is conjugated to the enzyme donor in such a way that aggregation with the enzyme acceptor is blocked if antibody binds to antigen. In the presence of analyte, less conjugate is bound by antibody, and enzyme activity is stimulated. In the absence of analyte, antibody binding to the conjugate prevents formation of the active enzyme.

Cells are cultured in a sterile, clear tissue culture treated microtitre plate, lysed and then the other components of the homogeneous CEDIA (antibody, enzyme-donor/ligand conjugate, enzyme acceptor monomer) are added dissolved in buffer containing the sequestration (cyclodextrin) agent. Optical density is measured and the concentration of analyte in the samples is determined by interpolation from a standard curve. Standards are added to empty wells of the same plate that are not used for growing cells

III Fluorescence Immunoassay Formats

Fluorescence polarization

Fluorescence polarization is a technique used to distinguish

from free analyte without the need for separation. In competitive assays, for small molecules, fluorescent-labelled antigens (e.g. using fluorescein, rhodamine or cyanine dye reagents) as tracer. At the signal generation and detection stage, a fluorimeter generates vertically polarized light at the excitation wavelength of the fluorophore.

The emitted light, at a lower wavelength because of Stokes' shift, is detected through a vertical polarizing filter. Because free tracer rotates at a very high speed, the emitted light is always in a different plane from the incident light, so the amount of light detected through the polarizing filter is minimal. However the tracer bound the much larger antibody molecule is restrained from rotating at such a high speed and the emitted light is in almost the same plane as the incident light. Cells are cultured in a sterile, clear tissue culture treated microtitre plate, lysed and then the other components of the homogeneous fluorescence assay (antibody, fluorescent-tagged antigen) are added dissolved in buffer containing the sequestration (cyclodextrin) agent. Fluorescence is measured and the concentration of analyte in the samples is determined by interpolation from a standard curve. Standards are added to empty wells of the same plate that are not used for growing cells.

Fluorescence resonance energy transfer (FRET)

Different fluorophores often have different activation and emission spectra (see Table 2). However, the activation peak of one fluorophore may overlap with the emission peak of another fluorophore. if the second fluorophore is placed in the immediate vicinity of the first, quenching of the fluorescent emission takes place through transfer of energy. This principle has been used in FRET by coupling one fluorophore to antibody and another to antigen. Binding of the antigen to antibody leads to close proximity and subsequent quenching. An alternative system involves two populations of antibodies raised against the same antigen,

labelled with two different fluorophores. Binding of the two antibodies gives rise to close proximity and then energy transfer between the two leading to quenching or reduction in fluorescence. Cells are cultured in a sterile, clear tissue culture treated microtitre plate, lysed and then the other components of the homogeneous fluorescence are added dissolved in buffer containing the sequestration (cyclodextrin) agent. Fluorescence is measured and the concentration of analyte in the samples is determined by interpolation from a standard curve. Standards are added to empty wells of the same plate that are not used for growing cells.

Table 2. The spectral properties of Cyanine Fluorescent Dyes

Fluorophore	Activation (nm)	Emission (nm)
Cy2	489	506
Cy3	550	570
Cy3.5	581	596
Cy5	649	670
Cy5.5	675	694
Cy7	743	767
FluorX	494	520

Time-resolved fluorescence

Background fluorescence is one of the main problems with the use of fluorescence immunoassay, and its presence can severely limit the use of these methods. However, background fluorescence present in most biological material has the short lifetime of a few nanoseconds. For fluorophores with long fluorescence lifetimes, it is possible to measure fluorescence at a time when virtually all the background fluorescence has disappeared. This is essentially the principle of time resolved fluorescence and this method can be used in combination with fluorescence energy

transfer and fluorescence polarization techniques.

IV) Other methods

The method described in this patent can be applied to other diverse homogeneous (non-separation) immunoassay techniques such as luminescence, nephelometry, latex agglutination assays and their variants.

EXAMPLES

A. PRELIMINARY EXPERIMENTS

Scintillation proximity radioimmunoassay for adenosine 3'5' cyclic monophosphate

General Assay Conditions

Measurement of adenosine 3'5'cyclic monophosphate (cAMP) was selected as a model system for studying lysis and measurement of intracellular molecules. A number of potential extractants were selected from among various surfactants known to lyse and liberate contents from eukaryotic cells. These lysis reagents were used in a series of experiments in combination with sequestrating reagents in order to determine optimal reagents to be used. Standard curves for cAMP were prepared in lysis and sequestrating reagents. Parameters such as assay sensitivity, standard curve working range and antigen: antibody binding were used to establish the most suitable reagents and optimal concentrations for both lysis and sequestrating agent. Standard curves were prepared as follows.

All assays were carried out in microtitre plates compatible with a microtitre plate beta scintillation counter. Standards (50µl; 0.2-12.8pmol/well), antisera (50µl; at 1:11000 dilution), tracer (50ul; 10000-

20000cpm) are added to anti-rabbit coated SPA beads (50µl; 20mg/ml). Non-specific binding was determined in the absence of specific rabbit antisera. SPA anti-rabbit reagent is placed onto a magnetic stirrer to ensure a homogeneous suspension before pipetting. All wells contained a total volume of 200µl. The plates were sealed and incubated at room temperature (15-30°C) for 15-20 hours. The amount of [^{125}I]cAMP bound to the SPA fluomicrospheres was determined by counting in a microtitre plate beta scintillation counter for 2 minutes.

Experiments were set up with:-

a) Working standards (4-256pmol/ml; 0.2-12.8pmol/well) were prepared in assay buffer (0.05M sodium acetate buffer containing 0.01% sodium azide) only (control) or assay buffer containing lysis reagent (e.g. DTAB, CPC or SDS; see experiment 1) at various concentrations.

b) Rabbit anti-cAMP sera prepared in assay buffer only (control) or assay buffer containing sequestrating agent at various concentrations depending on the experiment.

c) Radioactive tracer: adenosine 3' 5'-cyclic phosphoric acid 2'-O-succinyl-3-[^{125}I] iodotyrosine methyl ester prepared in assay buffer only (control) or assay buffer containing sequestrating agent at various concentrations depending on the experiment.

d) Donkey anti-rabbit IgG coupled to scintillation proximity fluomicrospheres prepared in assay buffer only (control) or assay buffer containing sequestrating agent at various concentrations depending on the experiment.

Experiment 1: Inhibition of antigen and antibody binding by several lysis reagents

Method

In these experiments, standard curves were prepared for

cAMP using the SPA immunoassay technique, described above, where working standards of cAMP were prepared in several lysis reagents at 1% (w/v) or 2% (w/v) final concentration. The detergents investigated included dodecyl trimethyl ammonium bromide (DTAB; Sigma Chemical Co. D8638), benzethonium chloride (BZC; Aldrich; B470-8), cetyl pyridinium chloride (CPC; Sigma Chemical Co; C9002), sodium dodecyl sulphate (SDS, Sigma chemical Co; L4509) and N-dodecyl-N,N-dimethyl-3-ammonio-1-propanesulphonate (DDAPS; Sigma Chemical Co.; D4516).

Results

Table 3a). The effect of several lysis reagents on antibody:antigen binding (zero concentration of sequestrant). These data shown are counts per minute (cpm) obtained on a TopCount™ microplate scintillation counter. Efficiencies of other multihead beta counters vary from this and may give different results. Non-specific binding (NSB) data was obtained in the absence of specific rabbit antisera.

Standard (pmol cAMP/well)	Control (no lysis reagent)	1% DTAB	1% BZC	1% CPC	1% SDS	1% DDAPS
NSB	113	130	111	121	118	181
Zero	2674	1429	951	466	1149	1109
0.2	2068	1118	644	270	1052	969
0.4	1952	1098	501	239	973	849
0.8	1770	1088	473	232	913	725
1.6	1463	977	426	147	859	610
3.2	1193	860	261	125	837	425
6.4	862	649	16	103	640	311
12.8	458	440	6	80	394	204

Table 3b). The effect of increased concentrations of several lysis reagents on antibody : antigen binding. These data shown are counts per minute (cpm) obtained on a TopCount microplate scintillation counter. Efficiencies of other multihead beta counters vary from this and may give different results. Non-specific binding (NSB) data was obtained in the absence of specific rabbit antisera.

Standards (pmol cAMP/ well)	Control (No detergent)	2% CPC	2% BZC	2% DTAB	2% DDAPS
NSB	117	133	117	145	129
Zero	2615	173	486	272	579
0.2	2100	133	308	231	416
0.4	1937	128	338	231	426
0.8	1786	108	222	201	429
1.6	1466	82	156	195	339
3.2	1202	77	143	179	303
6.4	867	73	96	170	215
12.8	459	50	70	93	109

Discussion

These data clearly demonstrates a significant reduction in antigen:antibody binding (cpm) where lysis reagents were included in the cAMP immunoassay.

Experiment 2: Restoration of antigen and antibody binding with the addition of sequestrant (2.5% alpha-cyclodextrin).

Method

In these experiments, standard curves for cAMP were prepared using the SPA immunoassay technique described above, where working standards of cAMP were prepared in 1% (w/v), 1.5% (w/v) or 2% (w/v) lysis reagents (DTAB, SDS or CPC). The control consisted of no lysis reagent or cyclodextrin added. The effect including 2.5% (w/v) alpha-cyclodextrin (alpha-CD) (Sigma Chemical Co.; C4642) on antigen:antibody binding was investigated. Here, tracer, antisera and SPA beads were prepared in assay buffer containing 2.5% (w/v) alpha-CD. A second control tested the effect of 2.5% (w/v) alpha-CD on the standard curve without addition of lysis reagent.

Results

Table 4a). The effect of lysis reagent (1% DTAB) on antigen : antibody binding with or without the addition of sequestrant. These data shown are counts per minute (cpm) obtained on a TopCount microplate scintillation counter. Efficiencies of other multihead beta counters vary from this and may give different results. Non-specific binding (NSB) data was obtained in the absence of specific rabbit antisera.

Standards (pmol cAMP/ well)	Control (No lysis reagent)	1% DTAB	2.5% CD only	1% DTAB 2.5% CD
NSB	186	182	155	140
Zero	3504	1586	3300	3361
0.2	3205	1575	2790	2931
0.4	2861	1573	2485	2638
0.8	2576	1305	1979	2228
1.6	1948	1097	1503	1724
3.2	1461	813	1058	1169
6.4	990	619	674	811
12.8	596	439	415	520

Table 4b). The effect of lysis reagent (1% SDS) on antigen : antibody binding with or without the addition of sequestrant. These data shown are counts per minute (cpm) obtained on a TopCount microplate scintillation counter. Efficiencies of other multihead beta counters vary from this and may give different results. Non-specific binding (NSB) data was obtained in the absence of specific rabbit antisera.

Standards (pmol cAMP/ well)	Control (No detergent)	1% SDS	2.5% cyclodextrin only	1% SDS plus 2.5% cyclodextrin
NSB	167	118	199	163
Zero	3147	1149	3145	3261
0.2	2335	1128	2358	2374
0.4	2179	1052	1979	2040
0.8	2080	973	1682	1851
1.6	1665	913	1499	1463
3.2	1269	859	1143	1100
6.4	857	640	754	813
12.8	411	394	383	416

Table 4c). The effect of lysis reagent (1 and 2% CPC) on antigen:antibody binding with or without the addition of sequestrant. These data shown are counts per minute (cpm) obtained on a TopCount microplate scintillation counter. Efficiencies of other multihead beta counters vary from this and may give different results. Non-specific binding (NSB) data was obtained in the absence of specific rabbit antisera.

Standards (pmol cAMP/ well)	Control (No detergent)	1% CPC	1% CPC 2.5% CD	2% CPC	2% CPC 2.5% CD
NSB	118	121	115	134	121
Zero	2615	466	2122	173	779
0.2	2100	270	1650	133	455
0.4	1937	239	1534	128	431
0.8	1786	232	1541	108	360
1.6	1466	147	1269	82	329
3.2	1202	125	1017	77	206
6.4	867	103	735	73	139
12.8	459	80	393	50	79

Table 4d). The effect of several lysis reagents on antigen:antibody binding with or without the addition of sequestrant. These data shown are counts per minute (cpm) obtained on a TopCount microplate scintillation counter. Efficiencies of other multihead beta counters vary from this and may give different results. Non-specific binding (NSB) data was obtained in the absence of specific rabbit antisera.

Standards (pmol cAMP/ well)	Control	1% DDAPS	1% DDAPS 2.5% CD	2% DDAPS	2% DDAPS 2.5% CD	2% DTAB	2% DTAB 2.5% CD
NSB	121	137	142	130	137	138	132
Zero	2989	906	2179	510	738	217	741
0.2	2453	762	1792	423	579	198	578
0.4	2279	719	1724	370	613	216	566
0.8	2072	689	1612	350	543	201	579
1.6	1734	559	1342	290	413	179	471
3.2	1346	446	1153	252	351	176	403
6.4	916	326	823	194	262	144	313
12.8	600	173	453	115	140	78	190

Table 4e). The effect of varying concentrations of alpha cyclodextrin on antibody:antigen binding in the presence of 1.5% lysis reagent. These data shown are counts per minute (cpm) obtained on a TopCount microplate scintillation counter. Efficiencies of other multihead beta counters vary from this and may give different results. Non-specific binding (NSB) data was obtained in the absence of specific rabbit antisera.

Standards (pmol cAMP/well)	**Control**	**1.5% DTAB only**	**1.5% DTAB 2.5% CD**	**1.5% DTAB 3% CD**
NSB	239	237	177	165
Zero	4623	819	4430	4306
0.2	4165	799	4069	3855
0.4	3612	713	3559	3629
0.8	3257	688	3369	2966
1.6	2550	513	2510	2356
3.2	1951	426	1991	1662
6.4	1368	376	1341	1220
12.8	922	264	849	753

Discussion

These data clearly demonstrates a significant restoration in antigen:antibody binding (cpm) where lysis reagents were added to the cAMP standards and where tracer, antisera and SPA beads were prepared in sequestration agent. Alpha-CD only had little impact on the standard curve.

Experiment 3: The optimal concentration of sequestrant

Method

In this experiment, standard curves for cAMP were prepared using the SPA radioimmunoassay where working standards of cAMP were prepared in 1% (w/v) DTAB. The effect of including 1% (w/v), 2% (w/v), 2.5% (w/v) and 5% (w/v) alpha-cyclodextrin on antigen:antibody binding was investigated. Here, tracer, antisera and SPA beads were prepared in assay buffer containing alpha-CD at the above concentrations. The control consisted of no lysis or cyclodextrin added to the assay.

Results

Table 5). The effect of lysis reagent and varying concentrations of sequestrant antigen : antibody binding. These data shown are counts per minute (cpm) obtained on a TopCount microplate scintillation counter. Efficiencies of other multihead beta counters vary from this and may give different results. Non-specific binding (NSB) data was obtained in the absence of specific rabbit antisera.

Standards (pmol cAMP/ well)	**Control**	**1% DTAB only**	**1% DTAB 1% CD**	**1% DTAB 2% CD**	**1% DTAB 2.5% CD**	**1% DTAB 5% CD**
NSB	172	130	121	179	165	140
Zero	3315	1429	1914	3250	3460	2980
0.2	3182	1118	1478	2849	3056	2490
0.4	2732	1098	1305	2525	2674	2145
0.8	2318	1088	1138	2143	2246	1800
1.6	1839	977	920	1639	1706	1372
3.2	1360	860	685	1245	1175	997
6.4	882	649	421	878	853	649
12.8	610	440	179	515	506	421

Discussion

These data clearly demonstrates a significant restoration in antigen:antibody binding (cpm) where lysis reagents were added to the cAMP standards and where tracer, antisera and SPA beads were prepared in sequestration agent. 1% (w/v) and 2% (w/v) alpha-CD restored binding to a limited degree. 5% (w/v) alpha-CD was inhibitory. 2.5% (w/v) alpha-CD gave optimal results.

Experiment 4: The optimal sequestrant

Method

In this experiment, standard curves for cAMP were prepared using the SPA immunoassay technique where working standards were prepared 1% (w/v) lysis reagent (DTAB). The effect of including 2.5% (w/v) alpha-cyclodextrin (CD) 2.5% (w/v) beta-cyclodextrin (beta-CD) (Sigma Chemical Co.; C4767) and 2.5% (w/v) gamma-cyclodextrin (gamma-CD) (Sigma Chemical Co.; 4892) on antigen:antibody binding was investigated. Here, tracer, antisera, and SPA beads were prepared in assay buffer containing the appropriate sequestration reagent. The control consisted of no lysis agent or cyclodextrin added to the assay.

Results

Table 6). The effect of adding different sequestrants on antigen:antibody binding. These data shown are counts per minute (cpm) obtained on a TopCount microplate scintillation counter. Efficiencies of other multihead beta counters vary from this and may give different results. Non-specific binding (NSB) data was obtained in the absence of specific rabbit antisera.

Standards (pmol cAMP/well)	Control	1% DTAB	1% DTAB 2.5% alpha CD	1% DTAB 2.5% beta CD	1% DTAB 2.5% gamma CD
NSB	212	130	173	103	172
Zero	4276	1429	4135	3225	4295
0.2	3752	1118	3604	2664	3650
0.4	3411	1098	3229	2360	3322
0.8	2788	1088	2548	1755	2270
1.6	2100	977	2005	1304	2066
3.2	1588	860	1480	966	1413
6.4	1104	649	1043	658	966
12.8	692	440	498	322	532

Discussion

The data demonstrates a significant restoration in antigen:antibody binding (cpm) where lysis agent was added to the cAMP standards, and where tracer, antisera and SPA beads were prepared in each of the sequestration reagents. Alpha and gamma cyclodextrin restored binding to an optimal degree, whereas beta-cyclodextrin was less

effective.

Overall Conclusions

These preliminary experiments established the utility of dodecyl trimethyl ammonium bromide (DTAB) as the preferred surfactant for cell lysis.

Similarly, in preliminary experiments, several sequestration reagents were evaluated including alpha-cyclodextrin, beta-cyclodextrin and gamma-cyclodextrin. Alpha-cyclodextrin was established as the preferred sequestration reagent.

B. THE OPTIMISED ASSAY SYSTEM

Example 1: Single-step extraction and measurement of adenosine 3', 5' cyclic monophosphate from forskolin-stimulated Chinese hamster ovary cells

Reagents, buffers and equipment

The following solutions were prepared:

1) Assay buffer; 0.05M sodium acetate buffer containing 0.01% (w/v) sodium azide.

2) Lysis reagent; DTAB (10% w/v) dissolved in assay buffer (useful for non-adherent cell lines).

3) Lysis reagent; DTAB (1% w/v) dissolved in assay buffer (useful for adherent cell lines such as Chinese Hamster Ovary Cells, this example) .

4) Sequestration reagent; alpha-cyclodextrin (2.5%w/v) dissolved in assay buffer.

5) Adenosine 3', 5' cyclic monophosphate (cAMP) standard; 512pmol, for the assay, used in the range 0.2-12.8pmol/well, prepared in

2ml of assay buffer containing 1%(w/v) DTAB to give 256pmol/ml (see below).

6) Radioactive tracer: adenosine 3', 5'-cyclic phosphoric acid 2'-O-succinyl-3-[^{125}I] iodotyrosine methyl ester prepared in assay buffer containing 2.5% alpha-cyclodextrin.

7) Rabbit anti-cAMP sera prepared in assay buffer containing 2.5% alpha-cyclodextrin.

8) Donkey anti-rabbit IgG coupled to scintillation proximity fluomicrospheres prepared in assay buffer containing 2.5% alpha-cyclodextrin.

Additional materials and equipment required are as follows.

i) Clear-bottomed microtitre plates with opaque walls, tissue culture treated plates (e.g.. Viewplates™, Packard or Cytostar-T plates, Amersham).

ii) Microplate scintillation counter.

iii) Plate sealers.

iv) Disposable polypropylene or polystyrene test tubes for preparing working standards.

v) Pipettes and pipetting equipment.

vi) Laboratory glassware.

vii) Distilled water.

viii) Vortex mixer.

ix) Magnetic stirrer.

x) 1% (w/v) trypan blue solution prepared in water.

xi) Haemocytometer

xii) HAM's culture media

xiii) Dimethylsulphoxide

xiv) Forskolin

xv) Cultured Chinese hamster ovary (CHO) cells at approximately 10^6 cells/ml.

Method

Chinese hamster ovary cells were grown in HAM's media containing 10% (v/v) foetal calf serum (FCS). For cAMP assays, cells were seeded into 96-well tissue culture plates (see materials) in HAM's media, as above, at 100µl/well (10^5 cells/well). The cells were cultured overnight at 37°C in a 95%air/5%CO_2 atmosphere. The next day, sequestration and lysis buffers (2.5% alpha-cyclodextrin and 1% DTAB in assay buffer) were prepared. Antisera (at 1/11000 dilution), tracer (10000-20000 cpm) and SPA beads (20mg/ml) were prepared in assay buffer containing 2.5% alpha-cyclodextrin. Working standards (4-256pmol/ml; 0.2-12.8pmol/microtitre well) were prepared in polypropylene tubes using assay buffer containing 1% DTAB. Fifty microlitres aliquots of each working standard were added to empty of wells of the same microtitre plate used for culturing cells. Forskolin (1mg) (to stimulate cAMP generation) was dissolved in DMSO (1ml), and diluted with HAM's media containing 10% FCS, to give various concentrations of forskolin from 1µM to 10µM. Two assay controls (blanks) were prepared in a similar manner. These consisted of a DMSO (assay) blank and a culture media (sample) only blank. The forskolin and blank solutions (100µl) were added to the cultured cells, and incubated at 37°C, as above, for 20 minutes. At this stage, a check was made that the cells remained viable using a trypan blue exclusion test. The culture media was aspirated from the stimulated walls (the cells remain adhered to the surface of the culture vessel), and the incubation terminated by the addition of lysis reagent (1% DTAB dissolved in assay buffer). A check for cell lysis was made with a second trypan blue exclusion test.

Fifty microlitres of primary antibody, tracer, and SPA beads (prepared in buffer containing 2.5% (w/v) alpha CD) , were added to standards and the samples in the 96-well culture plate. The plates were sealed and incubated overnight at room temperature. Following

incubation, the plates were transferred directly to a TopCount™ scintillation counter and radioactivity detected. Non-specific binding was determined in the absence of specific rabbit antisera. Cyclic AMP levels were determined using log/linear analysis with reference to the standard curve. Levels were estimated by interpolation.

This method is readily modified for extraction and measurement of cAMP in non-adherent cells (e.g.. HL60 cells). For this modified procedure, 10% (w/v) lysis reagent (10% DTAB dissolved in assay buffer) is added to the cultured cells to give a final concentration of 1% (w/v). The remainder of the method is as described above.

Results

Dose-response curves were prepared for the one-step *in situ* measurement of cAMP from forskolin stimulated CHO cells. Representative standard curves prepared on Viewplates and Cytostar-T plates are shown figures 1 and 2 respectively. In figure 3 , the effect of 1, 5 or 10μM forskolin on intracellular cAMP levels as measured by the one-step *in situ* measurement method on cells grown in 96-well plates is presented. CHO cells were seeded at a density of 100,000 cells/well and grown overnight in 96-well plates as described in the methods section. Cells were exposed to various concentrations of forskolin for 20 minutes and cAMP levels measured as described above. Basal levels of cAMP were approximately 10pmol/10^6 cells in the absence of added forskolin and were augmented by the increasing concentrations of forskolin.

Discussion

These data illustrate the utility of the invention described in this patent application, whereby intracellular molecules are measured in a concerted one-pot lysis and estimating immunoassay system. Indeed, the data presented in Figure 3 demonstrates lysis and measurement of cAMP

in forskolin-stimulated Chinese Hamster Ovary cells. The estimated levels of this cyclic nucleotide is in accordance with other published data whereby cAMP was determined by a more complex procedure (see Hancock *et al*, 1995). Furthermore, we have accumulated results in separate experiments whereby cAMP levels were measured with the method described in this patent application and compared with levels extracted and subsequently estimated with a traditional procedure (ethanol extraction procedure, Horton & Baxendale, 1995) (data not shown). The results, obtained with these two different methods, gave highly similar values in levels of intracellular cAMP.

Example 2: Single-step extraction and measurement of "total" cellular cAMP from forskolin-stimulated Chinese hamster ovary cells

This experiment describes the method for estimating levels of "total" cellular analyte. The procedure is applicable to cell culture systems and measures the intracellular fraction, and the component found in the cell culture supernatant. Accurate measurement of target analyte is achieved and the method has the advantage that aspiration or decantation of the cell culture media is not required. The technique is therefore useful for detection and measurement of molecules that are actively secreted into the extracellular fluid (see also Example 4).

Reagents, buffers and equipment

The following solutions were prepared.

1) Assay buffer; 0.05M sodium acetate buffer containing 0.01% (w/v) sodium azide.

2) Lysis reagent; DTAB (10% w/v) dissolved in assay buffer.

3) Lysis reagent; DTAB (1% w/v) dissolved in assay buffer.

4) Sequestration reagent; alpha-cyclodextrin (2.5% w/v) dissolved in assay buffer.

5) Adenosine 3', 5'-cyclic monophosphate (cAMP) standard; 512pmol (assay range, 0.2-12.8pmol/well) prepared in 2ml of assay buffer containing 1% (w/v) DTAB to give 256pmol/ml (see method).

6) Radioactive tracer: adenosine 3', 5'-cyclic phosphoric acid 2'-O-succinyl-3-[^{125}I] iodotyrosine methyl ester prepared in assay buffer containing 2.5% (w/v) alpha-cyclodextrin.

7) Rabbit anti-cAMP sera prepared in assay buffer containing 2.5% (w/v) alpha-cyclodextrin.

8) Donkey anti-rabbit IgG coupled to scintillation proximity fluomicrospheres prepared in assay buffer containing 2.5% (w/v) alpha-cyclodextrin.

Additional materials and equipment required are as follows:-

i) Clear-bottomed microtitre plates with opaque walls (tissue-culture treated) (e.g. Viewplates™, Packard or Cytostar-T plates, Amersham).

ii) Microplate scintillation counter.

iii) Plate sealers.

iv) Disposable polypropylene or polystyrene test tubes for preparing working standards.

v) Pipettes and pipetting equipment.

vi) Laboratory glassware.

vii) Distilled water.

viii) Vortex mixer.

ix) Magnetic stirrer.

x) 1% (w/v) trypan blue solution prepared in water.

xi) Haemocytometer

xii) HAM's culture media (Sigma; N-4888)

xiii) Dimethylsulphoxide

xiv) Forskolin

xv) Cultured Chinese hamster ovary (CHO) cells at approximately 10^6 cells/ml.

Method

Chinese hamster ovary (CHO) cells were cultured in HAM's media containing 10% (v/v) foetal calf serum (FCS). For cAMP assays, cells were seeded into clear-bottomed 96-well tissue-culture plates with opaque walls (tissue-culture grade; see materials) in HAM's media, (see experiment 5) at 40µl/well (between 10^4 and 10^6 cells/well). Cells were cultured overnight at 37°C in a 95% air/5% CO_2 atmosphere. The next day, sequestration and lysis buffers (2.5% alpha-cyclodextrin, 1% and 10% DTAB in assay buffer) were prepared. Antisera (at 1/11000 dilution), tracer (10000-20000 cpm) and SPA beads (20mg/ml) were reconstituted with assay buffer containing 2.5% (w/v) alpha-cyclodextrin. Working standards (4-256pmol/ml; 0.2-12.8pmol/microtitre well) were prepared in polypropylene tubes, using assay buffer containing 1% DTAB. 50µl of each working standard were added to empty wells of the microtitre plate used for culturing cells. Forskolin (1mg) (to stimulate cAMP generation) was dissolved in DMSO (1ml), and diluted with HAM's media containing 10% FCS, to give various concentrations of forskolin from 10µM to 1000µM (1µM to 100µM forskolin final concentration). To the cultured cells, 5µl aliquots of agonist or cell stimulant (in this case forskolin) was added directly to the cultured cells. The cells were incubated for 20 minutes at room temperature. The culture media was not aspirated or decanted after incubation. Two assay controls (blanks) were prepared in a similar manner. These consisted of a DMSO (assay) blank and a culture media (sample) only blank. At this stage, a check was made using a cell viability (trypan blue exclusion) test. To the stimulated cells, 5µl of cell lysis reagent (10% DTAB in assay buffer) was added. The final volume was 50µl, each well containing 1% cell lysis reagent (final concentration). The

cells were agitated after cell lysis reagent was added by vigorous, successive pipetting. The plate was incubated for 5 minutes at room temperature. Cell lysis was checked with a second trypan blue exclusion test. The extracted cAMP was immediately processed for measurement with the SPA radioimmunoassay. (In this example, aliquots were not transferred to a second plate for assay.)

DTAB (100 µl, 1% w/v in assay buffer) was added to the non-specific binding wells. DTAB (50 µl, 1% w/v in assay buffer) was added to the zero standard wells. 50 µl of each standard (prepared in assay buffer containing 1% DTAB) was added to the appropriate wells. 50 µl of primary antibody, tracer, and SPA beads (prepared in buffer containing 2.5% (w/v) alpha-cyclodextrin), were added to the standards and samples. The plate was sealed and incubated overnight at room temperature. Following incubation, the plate was transferred directly to a TopCount™ scintillation counter and radioactivity detected. Non-specific binding was determined in the absence of specific rabbit antisera. Cyclic AMP levels were determined using log/linear analysis with reference to the standard curve. Levels were estimated by interpolation.

This method is readily modified for extraction and measurement of cAMP in non-adherent cells (e.g. HL60 cells). For this modified procedure, 10% (w/v) lysis reagent (10% DTAB dissolved in assay buffer) was added to the cultured cells to give a final concentration of 1% (w/v). There was no centrifugation or decantation and aspiration steps to remove the cell culture supernatant. The remainder of the method is as described above.

Results

The effect of 1µM to 100µM forskolin on "total" cellular cAMP levels in cultured CHO cells, is presented in Figure 4. In this experiment, CHO cells were seeded at a density of 100,000 cells/well and grown

overnight in 96-well plates as described in the methods section. Cells were exposed to various concentrations of forskolin for 20 minutes and total cAMP levels measured as described above. Basal levels of cAMP were less than 10pmol/10^6 cells in the absence of forskolin. Total cellular cAMP levels rose to over 170pmol/10^6 cells in the presence of 100μM forskolin.

Example 3: Direct measurement of intracellular Interleukin-6 by enzyme-linked immunosorbent assay

This experiment describes a simple and direct method for the measurement of intracellular levels of Interleukin-6 (a cytokine). Unlike the previous examples for cAMP, the method described here is a two-stage process whereby cells are cultured, stimulated and lysed on a conventional tissue plate, and an aliquot of lysate is transferred to a second plate for assay.

Endothelial cells were stimulated with Interleukin-1β (IL-1β) overnight and lysed with DTAB. The lysate was analysed for the presence of Interleukin-6 (IL-6) by ELISA. The critical component of the ELISA (the biotinylated antibody) was prepared in buffer containing the sequestrant (3% w/v alpha-cyclodextrin). The method is quick, easy and sensitive enough to require only a few cells (<10^5) per well.

Reagents, buffers and equipment

1) Human Interleukin-6 ELISA kit, Amersham, RPN 2754
2) Alpha-cyclodextrin, USB, 13979
3) DTAB, Sigma, D8638
4) IL-1β , Amersham, ARM 17005
5) ECV304 cells, a human endothelial cell line derived from a new-born Japanese female.

Additional materials and equipment are as follows.

a) Standard 96-well tissue culture plates

b) Disposable test tubes for preparing working standards

c) Pipettes and pipetting equipment

d) Laboratory glassware

e) Distilled water

f) Vortex mixer

g) Magnetic stirrer

h) 1% (w/v) trypan blue solution prepared in water

i) Haemocytometer

j) M-199 media, Sigma, M-7653

k) Microtitre plate washer

l) Microtitre plate reader

Method

ECV304 cells were cultured in M-199 media containing 10% (v/v) foetal calf sera (FCS). For IL-6 assays, cells were seeded into standard 96-well tissue culture plates in 100µl volumes (between 10^5 - 10^6 cells/ml). Cells were cultured overnight at 37°C in a 95% air/5% CO_2 atmosphere. On day 2 working solutions of IL-1β (2-500pg/ml; final concentration) were prepared in M-199 media in order to stimulate the production of IL-6. A culture media blank was prepared with cultured cells grown in the absence of IL-1β. Cells were again cultured overnight at 37°C in a 95% air/5% CO_2 atmosphere. On day 3, a 1% (w/v) solution of DTAB was prepared in standard diluent. This reagent was used to lyse the cells and for the preparation of working standards. After overnight incubation, the cell culture supernatants were decanted (the cells remain adhered to the surface of the culture vessel). The cells were gently washed X3 with phosphate buffered saline. After the third wash, the cells were checked to ensure none were lost. 100µl of the lysis reagent (1% DTAB in standard diluent) was added to the cells. Cell lysis was checked with trypan blue exclusion.

Working standards (10.24-400 pg/ml IL-6) were prepared in polypropylene tubes using assay buffer containing 1% (w/v) DTAB. The biotinylated antibody was prepared in buffer containing 3% (w/v) alpha-cyclodextrin. 50µl of biotinylated antibody (containing cyclodextrin) was added to the anti-IL-6 coated plate. 50µl of working standard and cell lysate was pipetted into separate wells of the anti-IL-6 coated plate. Non-specific binding was measured in the absence of IL-6 (zero IL-6 standard). The order of addition of biotinylated antibody and samples/ standards is not critical to the procedure. However, in this example, improved results were obtained when the biotinylated antibody was added to the anti-IL-6 coated plate before the standards or samples. The anti-IL-6 plate, containing biotinylated antibody and standards/samples, was incubated for 2 hours at room temperature. The plate was washed thoroughly, followed by the addition of 100µl/well of diluted (30µl of concentrate to 12ml of streptavidin dilution buffer) peroxidase-labelled streptavidin. The plate was incubated at room temperature for 30 minutes followed by thorough washing. 100µl of TMB substrate was added to each well of the plate, followed by a 30 minute incubation at room temperature. The reaction was terminated by the addition of 100µl/well sulphuric acid. The optical density was determined with a microtitre plate spectrophotometer set at 450nm. Interleukin-6 levels were determined using log/linear analysis with reference to a standard curve. Levels were estimated by interpolation.

Results

The effect of including lysis reagent and sequestrant on the IL-6 ELISA system is presented in Figures 5 & 6. Figure 5 shows an inhibition of antibody binding when 1% DTAB is added to the IL-6 assay. Binding was restored upon the addition of 3% (w/v) alpha-cyclodextrin (Figure 6).The results of stimulating ECV304 cells with IL-1β, and the measurement of intracellular IL-6 is shown in Figure 7. Basal levels of IL-6

were less than 30pg/10^6 cells in the absence of IL-1β. Intracellular IL-6 levels rose to over 400pg/10^6 cells in the presence of 500 pg/ml IL-1β.

Example 4: Measurement of "total" cellular Interleukin-6 from IL-1β stimulated ECV304 cells

This experiment describes a method for measurement of "total" cellular Interleukin-6. The procedure is applicable to cell culture systems and is suitable for the measurement of molecules (such as cytokines) that are secreted into cell culture fluids.

Reagents, buffers and equipment

1) Human Interleukin-6 ELISA kit, Amersham, RPN 2754
2) Alpha-cyclodextrin, USB, 13979
2) DTAB, Sigma, D8638
3) IL-1β , Amersham, ARM 17005
4) ECV 304 cells, a human endothelial cell line derived from a new-born Japanese female.

Additional materials and equipment are as follows.

a) Standard 96-well tissue culture plates
b) Disposable test tubes for preparing working standards
c) Pipettes and pipetting equipment
d) Laboratory glassware
e) Distilled water
f) Vortex mixer
g) Magnetic stirrer
h) 1% (w/v) trypan blue solution prepared in water
i) Haemocytometer
j) M-199 media
k) Microtitre plate washer
l) Microtitre plate reader

Method

ECV304 cells were cultured in M-199 media containing 10% (v/v) foetal calf sera (FCS). For IL-6 assays, cells were seeded into standard 96-well tissue culture plates in 100µl volumes (between 10^5 - 10^6 cells/ml). Cells were cultured overnight at 37°C in a 95% air/5% CO_2 atmosphere. On day 2, working solutions of IL-1β (2-500pg/ml, final concentration) were prepared in M-199 media in order to stimulate the production of IL-6. A culture media blank was prepared with cells in the absence of IL-1β. Cells were again cultured overnight at 37°C in a 95% air/5% CO_2 atmosphere. On day 3, sequestration and lysis buffers (3% alpha-cyclodextrin, 1% and 10% DTAB) were prepared. The lysis reagents were prepared in standard diluent. The 10% DTAB solution was used for cell lysis, the 1% DTAB solution for the preparation of working standards.

10µl of cell lysis reagent (10% DTAB) were added to the stimulated cells. The cells were agitated and the plate incubated for 5 minutes at room temperature. The final volume in the wells was 110µl. Cellular lysis was checked with trypan blue exclusion. The "total" cellular IL-6 was measured immediately with ELISA.

Working standards (10.24-400 pg/ml IL-6) were prepared in polypropylene tubes with assay buffer containing 1% (w/v) DTAB. The biotinylated antibody was prepared in buffer containing 3% (w/v) alpha-cyclodextrin. 50µl of biotinylated antibody (containing cyclodextrin) was added to an anti-IL-6 coated plate. 50µl of working standard and "total" cell lysate was pipetted into separate wells of the anti-IL-6 coated plate. Non-specific binding was measured in the absence of IL-6 (zero IL-6 standard). The order of addition of biotinylated antibody and samples/ standards is not critical to the procedure. However, in this example, improved results were obtained when the biotinylated antibody was added to the anti-IL-6 coated plate before the standards or samples. The anti-IL-6 plate, containing biotinylated antibody standards and samples, was incubated for

2 hours at room temperature. The plate was washed thoroughly, followed by the addition of 100µl/well of diluted (30µl of concentrate added to 12ml of streptavidin dilution buffer) peroxidase-labelled streptavidin. The plate was incubated at room temperature for 30 minutes followed by thorough washing. One hundred microlitres of TMB substrate was added to each well, followed by a 30 minute incubation at room temperature. The reaction was terminated by the addition of 100µl/well sulphuric acid. The optical density was determined in each well with a microtitre plate spectrophotometer set at 450nm. Total cellular Interleukin-6 levels were determined using log/linear analysis with reference to the standard curve. Levels were estimated by interpolation.

Results

The effect of stimulation of ECV304 cells with IL-1β, and the measurement of intracellular, total cellular, and IL-6 measured in the cell culture supernatant, is presented in Figure 8. Compared with the cell culture supernatant, significantly higher levels of IL-6 was measured in the "total" lysate fraction.

Experiment 5: Radioreceptor binding assay for D-*myo*-inositol 1,4,5-trisphosphate (IP_3)

1. Established methods for preparing cells for measurement of inositol 1,4,5-trisphosphate (IP_3) include acid extraction procedures, processes which require careful neutralisation with alkali before assay. The technique described here is based on competition of [^{3}H] inositol 1,4,5-trisphosphate (the tracer) with unlabelled IP_3 in the sample or standard to a binding protein prepared from bovine adrenal cortex. As in previous experiments, DTAB is used as a lysis reagent and alpha-cyclodextrin as the sequestrant. Clearly, the method is applicable to the intracellular

measurement of IP_3, and has a number of advantages over traditional techniques for IP_3 extraction before to assay.

Reagents, buffers and equipment

1. D-myo-inositol 1,4,5-trisphosphate (IP_3) Radioreceptor kit, Amersham, TRK1000
2. 2) Alpha-cyclodextrin, USB, 13979
3. 3) DTAB, Sigma, D8638
4. 4) 10% (v/v) acetic acid
5. 5) 0.15M sodium hydroxide

Additional materials and equipment required are as follows.

a) Pipettes and pipetting equipment
b) Polypropylene test tubes
c) Distilled water
d) Vortex mixer
e) Refrigerated centrifuge
f) β-scintillation counter
g) Scintillant
h) Counting vials
i) Ice bath
j) Decantation racks

Method

Bovine adrenal glands were removed from animals and stored at-20°C before preparing the binding protein. The cortex was dissected from the adrenal glands, homogenised in $NaHCO_3$ (20mM) with dithiothreitol (1mM), and the homogenate centrifuged at 5000g for 15 minutes. The supernatant was centrifuged at 35000g for 20 minutes, the resulting pellet resuspended in the homogenisation buffer and centrifuged

again at 35000g for 20 minutes. The final pellet was resuspended in homogenisation buffer at a protein concentration of between 20-40mg/ml.

For the assay, aliquots (100µl) of the bovine adrenal cortex microsome preparation were incubated in 100µl of 0.1M Tris buffer (pH9.0), containing 4mM EDTA and 4mg/ml bovine serum albumin. Incubations were carried out for 15 minutes in a final volume of 0.4ml with [^{3}H] inositol 1,4,5-trisphosphate (100µl; 6000cpm) sample or standard (100µl; 0.19-25pmol inositol 1,4,5-trisphosphate / assay tube). Non-specific binding was determined in the presence of 1nmol inositol 1,4,5 - trisphosphate / tube. Incubations were terminated by centrifugation (12000g) (10 minutes at 4°C) and removal of the supernatant by gentle decantation. Particulate bound radioactivity was analysed, after suspension in 0.15M sodium hydroxide followed by neutralisation with 10% acetic acid, by liquid scintillation counting

Experiments were set up with:-

1) Working standards (0.19-25pmol IP_3 / tube) were prepared in water (control) or, in water containing lysis reagent (0.5%w/v).

2) The binding protein, prepared as above (control) or, in homogenisation buffer containing 2% (w/v) alpha-cyclodextrin.

3) Radioactive tracer prepared in water (control) or, in water containing 2% (w/v) alpha-cyclodextrin.

Results

The effect of adding lysis reagent to the IP_3 standards and sequestrant to the tracer and binding protein is shown in Figure 9. In standard curves where lysis reagent (0.5%w/v DTAB) was included, there was a total inhibition of binding to the receptor preparation. Binding was restored when alpha-cyclodextrin (2% final w/v) was added to both the tracer and the adrenal cortex preparation.

Example 5: Direct Measurement of Intracellular Prostaglandin E_2 in Mouse 3T3 Cells

This experiment describes a simple and convenient method for the direct measurement of intracellular levels of prostaglandin E_2 from stimulated Mouse Swiss 3T3 Albino embryo fibroblast cells. Prostaglandin E_2 (PGE_2) is a product of arachidonic acid metabolism and the cyclooxygenase pathway. The method described here involves a two-stage process where cells are cultured, stimulated and lysed on a conventional tissue-culture plate and an aliquot of lysate is transferred to a second plate for measurement with a competitive enzymeimmunoassay (EIA) technique. Mouse 3T3 cells were stimulated with the calcium ionophore A23187 for 5 minutes, washed, and lysed with DTAB. The lysate was analysed for the presence of PGE_2 with EIA. The critical components of the assay (the PGE_2 peroxidase conjugate and the PGE_2 antiserum) were prepared in buffer containing the sequestrant (2.5% alpha-cyclodextrin). The method is very quick, easy to carry out and sensitive enough to require only a few cells ($<10^5$) per well.

Reagents, buffers and equipment

1. Prostaglandin E_2 enzymeimmunoassay (EIA) kit, Amersham, RPN 222
2. Alpha-cyclodextrin, USB, 13979
3. DTAB, Sigma, D-8638
4. Calcium ionophore A23187, Sigma, C-7522
5. Mouse 3T3 cells, ECACC
6. DMEM media, Sigma, D-6546

Additional materials and equipment are as follows:-

a) Standard 96-well tissue culture plates

b) Disposable test tubes for preparing working standards

c) Pipettes and pipetting equipment

d) Laboratory glassware

e) Distilled water

f) Vortex mixer

g) Magnetic stirrer

h) 1% (w/v) Trypan blue solution prepared in water

i) Haemocytometer

j) Microtitre plate washer

k) Microtitre plate reader

Method

Mouse 3T3 cells were cultured in DMEM media containing 10% (v/v) foetal calf sera (FCS). For PGE_2 assays, cells were seeded into standard 96-well tissue culture plates in 100µl volumes (between 10^5-10^6 cells/ml). Cells were cultured overnight at 37°C in a 95% air/5% CO_2 atmosphere. At this stage a check was made to ensure cells remained viable with Trypan blue exclusion.

On day 2, working solutions of A23187 (1-100µM final concentration) were prepared in DMEM media in order to stimulate the production of PGE_2. Two controls were prepared (a DMSO and a culture media only control). Here cells were cultured in the absence of A23187. Test cell cultures were stimulated with the calcium ionophore A23187 for 5 minutes at room temperature. The culture supernatant was decanted, cells were washed with phosphate buffered saline and lysed with 100µl/well 0.5% (w/v) DTAB prepared in assay buffer. A check for cell lysis was made with a second Trypan blue exclusion test.

Working PGE_2 standards (2.5-320 pg/well) were prepared in polypropylene tubes with assay buffer containing 0.5% (w/v) DTAB. The PGE_2 antibody and PGE_2 conjugate were prepared with assay buffer containing 2.5% (w/v) alpha-cyclodextrin. 50µl of working standard and cell

lysate were pipetted into separate wells of a goat anti-mouse IgG coated plate. Non-specific binding was measured in the absence of PGE_2 antisera. Zero standard PGE_2 consisted of assay buffer containing 0.5 % (w/v) DTAB only. 50µl of antisera and 50µl conjugate (prepared in assay buffer containing 2.5% (w/v) alpha-cyclodextrin) were pipetted into the appropriate test wells (containing standards and sample cell lysates). The plates were incubated for 1 hour at room temperature with constant shaking, followed by thorough washing. 150µl of TMB substrate was added to all wells and incubated for 30 minutes at room temperature. The reaction was terminated by the addition of 100µl/well sulphuric acid. The optical densities were determined with a microtitre plate spectrophotometer set at 450nm. Intracellular PGE_2 levels were determined using log/linear analysis with reference to a standard curve. Levels were estimated by interpolation.

Results

The results of stimulating 3T3 cells with the calcium ionophore A23187, and the direct measurement of intracellular PGE_2 are shown in Figure 10. Basal levels of PGE_2 were approximately 50pg/10^6 cells in the absence of A23187. Intracellular PGE_2 levels rose to over 400pg/10^6 cells in the presence of 100µM A23187.

LEGEND TO FIGURES

Figure 1. Representative standard curve for the concerted one-pot extraction and immunoassay method for the estimation of adenosine 3'5' cyclic monophosphate from cultured Chinese Hamster Ovary cells.

Assay curves were prepared on Viewplates (Packard) as described in the methods section.

The percent B/B_0 function was calculated from the following

formula:-

[(standard counts per minute)-(non-specific binding counts per minute)] divided by [(zero standard counts per minute)-(non-specific binding counts per minute)] multiplied by 100%.

Typically, counts per minute values for the zero dose were 2200. The counts per minute values obtained in the absence of specific antibody (non-specific binding wells) were approximately 200. The values in the absence of tracer were usually less than 25cpm.

Figure 2. Representative standard curve for the concerted one-pot extraction and immunoassay method for the estimation of adenosine 3'5' cyclic monophosphate from cultured Chinese Hamster Ovary cells.

Assay curves were prepared on Cytostar-T as described in the methods section.

The percent B/B_0 function was calculated from the following formula:-

[(standard counts per minute)-(non-specific binding counts per minute)] divided by [(zero standard counts per minute)-(non-specific binding counts per minute)] multiplied by 100%.

Typically, counts per minute values for the zero dose were 2200. The counts per minute values obtained in the absence of specific antibody (non-specific binding wells) were approximately 200. The values in the absence of tracer were usually less than 25cpm.

Figure 3. Forskolin-stimulated cAMP generation from cultured Chinese Hamster Ovary Cells as determined by the one-pot extraction SPA radioimmunoassay method.

CHO cells were seeded at a density of 100,000 cells per well and grown overnight to confluence in clear-bottomed Cytostar-T or

Viewplates as described under the methods section. Cells were exposed to various concentrations of forskolin and cAMP extracted and levels determined with the SPA radioimmuunoassay procedure. Basal levels of cAMP were less than 10pmoles/10^6 cells which significantly increased on stimulation by forskolin.

Figure 4. "Total" cellular cAMP measurement from cultured Chinese hamster ovary cells

Chinese hamster ovary cells were seeded at a density of 100,000 cells per well and cultured overnight to confluence in Viewplates as described under the methods section. Cells were exposed to various concentrations of forskolin (1μM-100μM) for 20 minutes at room temperature. Cells were lysed in situ by addition of lysis reagent (10% DTAB added, 1% final concentration). The cell culture supernatant was not removed before the cell lysis step. cAMP levels were measured with the SPA radioimmunoassay procedure. Basal levels of cAMP were less than 10pmoles/10^6 cells, total cellular cAMP levels rose significantly when cells were stimulated with forskolin.

Figure 5. Inhibition of binding in the IL-6 ELISA with lysis reagent

Interleukin- 6 standards (10.24-400pg/ml) were prepared in standard diluent either in the presence (□) or absence(●) of lysis reagent (1%w/v DTAB). Aliquots (50μl) of biotinylated antibody (zero cyclodextrin), were added to the anti-IL-6 coated plate followed by standard (50μl). The plate was incubated for 2 hours at room temperature, and the optical density measured as described under the methods section.

Figure 6. Restoration of binding in the IL-6 ELISA with sequestrant

Interleukin-6 standards (10.24-400pg/ml) were prepared in standard diluent either in the presence (□) or absence(●) of lysis reagent

(1%w/v DTAB). Aliquots (50μl) of biotinylated antibody, prepared in the presence (□) or absence(●) of cyclodextrin (3% w/v), were added to the anti-IL-6 coated plate followed by standard (50μl). The plate was incubated for 2 hours at room temperature, and the optical density measured as described under the methods section.

Figure 7. Intracellular measurement of IL-6 from IL-1β stimulated ECV304 cells

ECV304 cells were seeded at a density of 100,000 cells per well and cultured overnight to confluence in standard 96-well tissue culture plates as described under the methods section. Cells were exposed to various concentrations of IL-1β (2-500pg/ml) overnight and the supernatant decanted. The cells were washed thoroughly and 100μl of lysis reagent (1% DTAB) added. Aliquots (50μl) of cell lysate were transferred to a second 96-well plate coated with anti-IL-6 antibody for assay. Levels of IL-6 were measured in the samples with ELISA (the biotinylated antibody was prepared in the presence of 3% (w/v) alpha-cyclodextrin).

Figure 8. Interleukin-6 measurement from ECV304 cells

ECV304 cells were seeded at a density of 100,000 cells per well and cultured overnight to confluence in standard 96-well tissue culture plates as described under the methods section. Cells were exposed to IL-1β overnight and IL-6 measured in the intracellular fraction (see experiment 7), cell culture supernatant (with a traditional ELISA technique), and in the "total" cellular fraction. For the total cellular assay, cells were lysed in situ by addition of lysis reagent (10% DTAB added, 1% final concentration). The cell culture supernatant was not removed before the cell lysis step. Aliquots (50μl) of "total" cellular lysate were transferred to a second 96-well plate coated with anti-IL-6 antibody for assay. Levels of IL-6 were

measured in the samples with ELISA (the biotinylated antibody was prepared in the presence of 3% (w/v) alpha-cyclodextrin).

Figure 9. Standard curves for the IP_3 radioreceptor assay

Increasing concentrations of inositol 1,4,5-trisphosphate were allowed to compete with [^{3}H] inositol 1,4,5-trisphosphate for binding to the bovine adrenal cortex binding protein for 15 minutes at 4^{o}C. Curves were prepared in the absence of both lysis reagent and sequestrant (control,●),with lysis reagent only (□), with sequestrant only (▼), and with both lysis reagent and sequestrant (☆). The lysis reagent totally inhibited specific binding to the receptor preparation. Binding was restored when tracer and binding protein were prepared with sequestrant.

Figure 10. Intracellular PGE_2 measurement from 3T3 cells

3T3 cells were seeded at a density of 100,000 cells per well and cultured overnight to confluence in standard 96-well tissue culture plates as described under the methods section. Cells were exposed to various concentrations of calcium ionophore A23187 (1-100μM) for 5 minutes. and the supernatant decanted. The cells were washed thoroughly and 100μl of lysis reagent (0.5% DTAB) added. Aliquots (50μl) of cell lysate were transferred to a second 96-well plate coated with goat anti-mouse IgG for assay. The lysate was analysed for the presence of (PGE_2) by EIA. The critical components of the assay (the PGE_2 peroxidase conjugate and the PGE_2 antiserum) were prepared in buffer containing the sequestrant (2.5% alpha-cyclodextrin).

CLAIMS

1. A method of assaying for an analyte which method comprises the steps of:

i) mixing a sample of cells possibly containing the analyte with a cell lysis reagent to provide a cell lysis fluid,

ii) mixing the cell lysis fluid with reagents, including a specific binding partner of the analyte for binding to the analyte, for performing a specific binding assay for the analyte,

iii) and mixing the cell lysis fluid with a sequestrant for the cell lysis reagent, whereby the binding of step ii) is performed in the presence of the sequestrant.

2. A method as claimed in claim 1, wherein the cell lysis reagent is a detergent.

3. A method as claimed in claim 1, wherein the sequestrant is a cyclodextrin.

4. A method as claimed in claim 3, wherein the amount of sequestrant is in the range of 1 - 5% of the binding reaction mixture.

5. A method as claimed in claim 1, wherein steps i), ii) and iii) are all performed in a single reaction vessel.

6. A method as claimed in claim 1, wherein multiple assays are performed in parallel in wells of a multiwell plate.

7. A method as claimed in claim 1, wherein the cells are cultured in a vessel and are lysed in that vessel for assaying the analyte in that vessel.

8. A method as claimed in claim 1, wherein the assay of step ii) is a homogenous assay.

9. A method as claimed in claim 1, wherein the assay of step ii) is a scintillation proximity assay.

10. A method as claimed in claim 1, wherein the specific binding assay of step ii) is an immunoassay.

11. A method as claimed in claim 1, wherein the analyte is adenosine-3',5'-cyclic monophosphate, the cell lysis reagent is dodecyl trimethyl ammonium bromide and the sequestrant is α-cyclodextrin.

12. A method as claimed in claim 1, wherein the cells have been maintained in a culture medium, and step i) is performed in the presence of the culture medium.

13. A method as claimed in claim 1, wherein the intracellular or the total (intracellular plus extracellular) concentration is measured of an analyte selected from adenosine-3',5'-cyclic monophosphate, interleukin-6 and prostaglandin E_2.

14. A kit, suitable for assaying for an analyte by the method as claimed in claim 1, comprising: a detergent; a sequestrant for the detergent; a specific binding partner of the analyte; a tracer; and separation means for separating bound tracer from unbound tracer.

ABSTRACT

IN-SITU CELL EXTRACTION AND ASSAY METHOD

A method of assaying for an analyte comprises the steps of:

i) mixing a sample of cells possibly containing the analyte with a cell lysis reagent to provide a cell lysis fluid,

ii) mixing the cell lysis fluid with reagents, including a specific binding partner of the analyte for binding to the analyte, for performing a specific binding assay for the analyte,

iii) and mixing the cell lysis fluid with a sequestrant for the cell lysis reagent, whereby the binding of step ii) is performed in the presence of the sequestrant.

Fig.1.

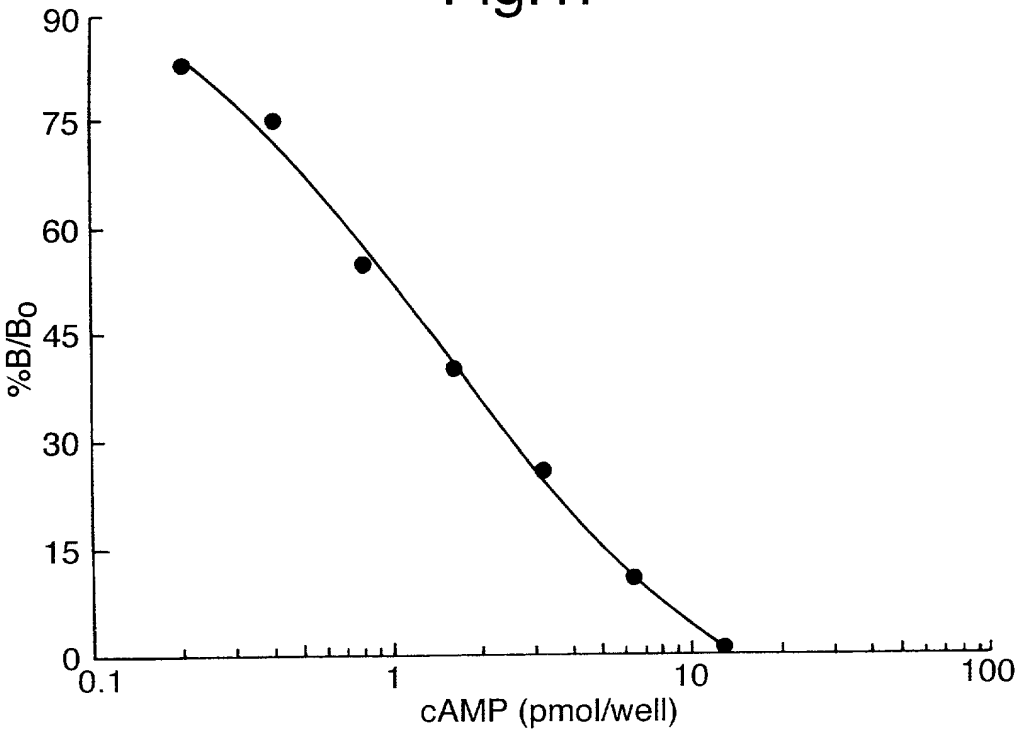

90
75
60
45
30
15
0
%B/B0
0.1
1
10
100
cAMP (pmol/well)

Fig.2.

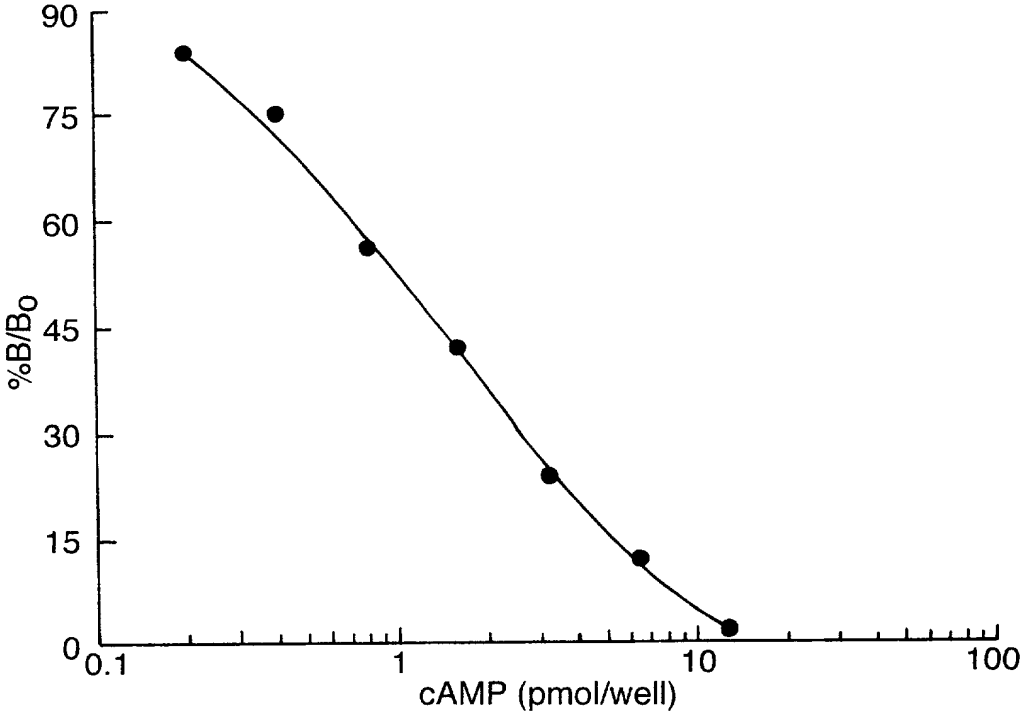

90
75
60
45
30
15
0
%B/B0
0.1
1
10
100
cAMP (pmol/well)

Fig.3.

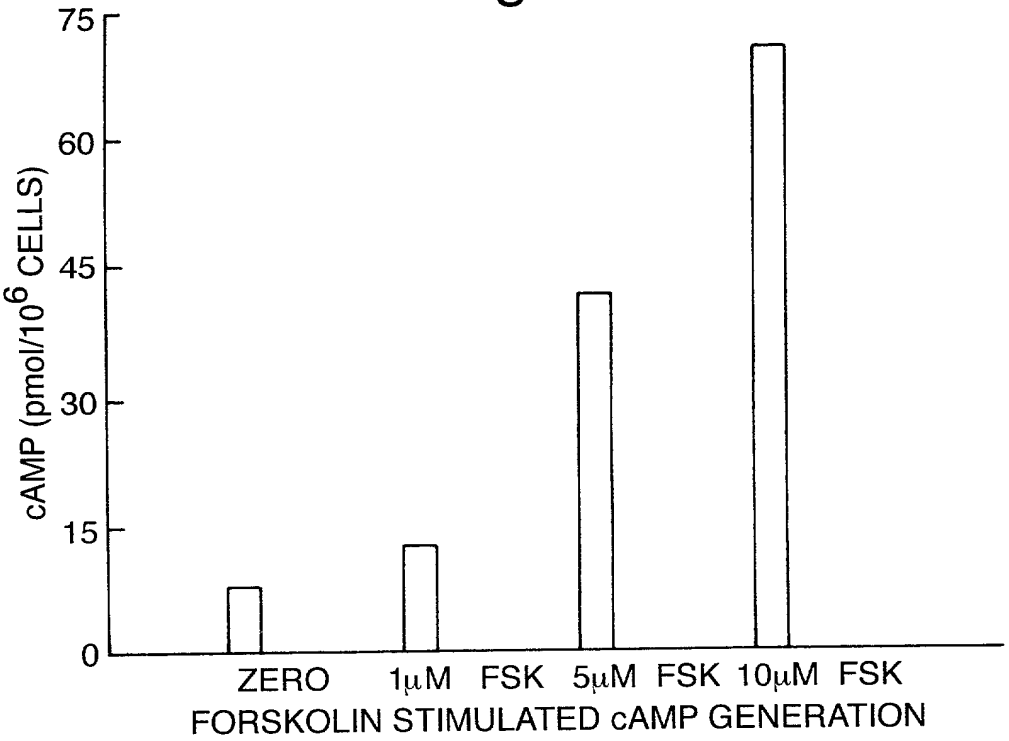

75
60
45
30
15
0
cAMP (pmol/10^6 CELLS)
ZERO
1μM FSK
5μM FSK
10μM FSK
FORSKOLIN STIMULATED cAMP GENERATION

Fig.4.

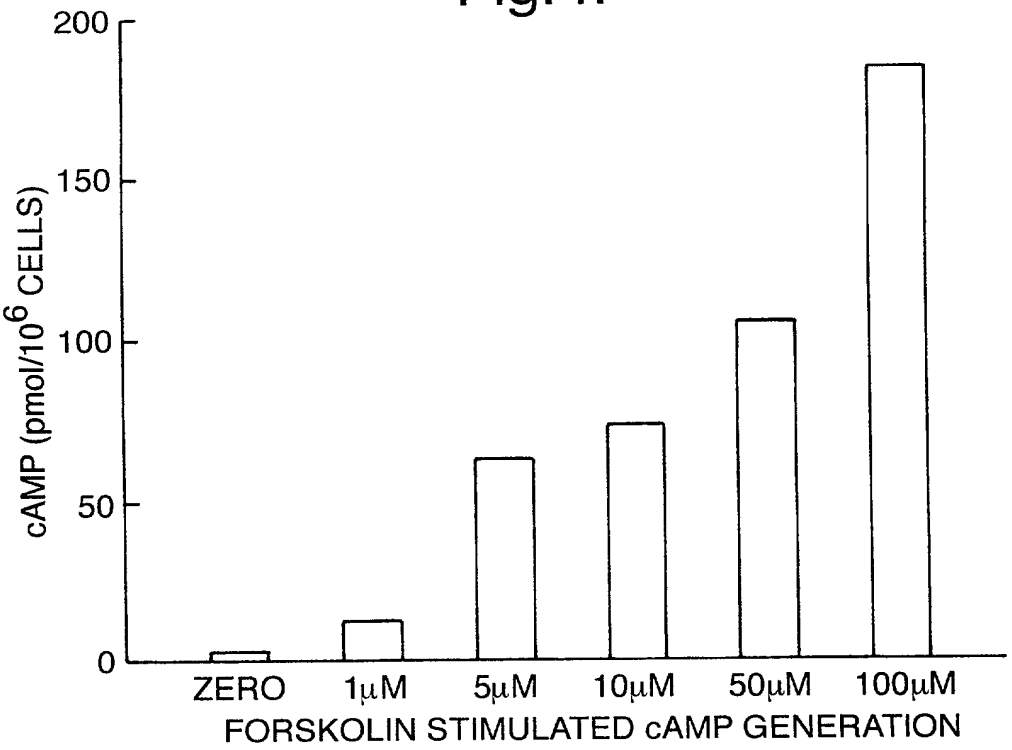

200
150
100
50
0
cAMP (pmol/10^6 CELLS)
ZERO
1μM
5μM
10μM
50μM
100μM
FORSKOLIN STIMULATED cAMP GENERATION

Fig.5.

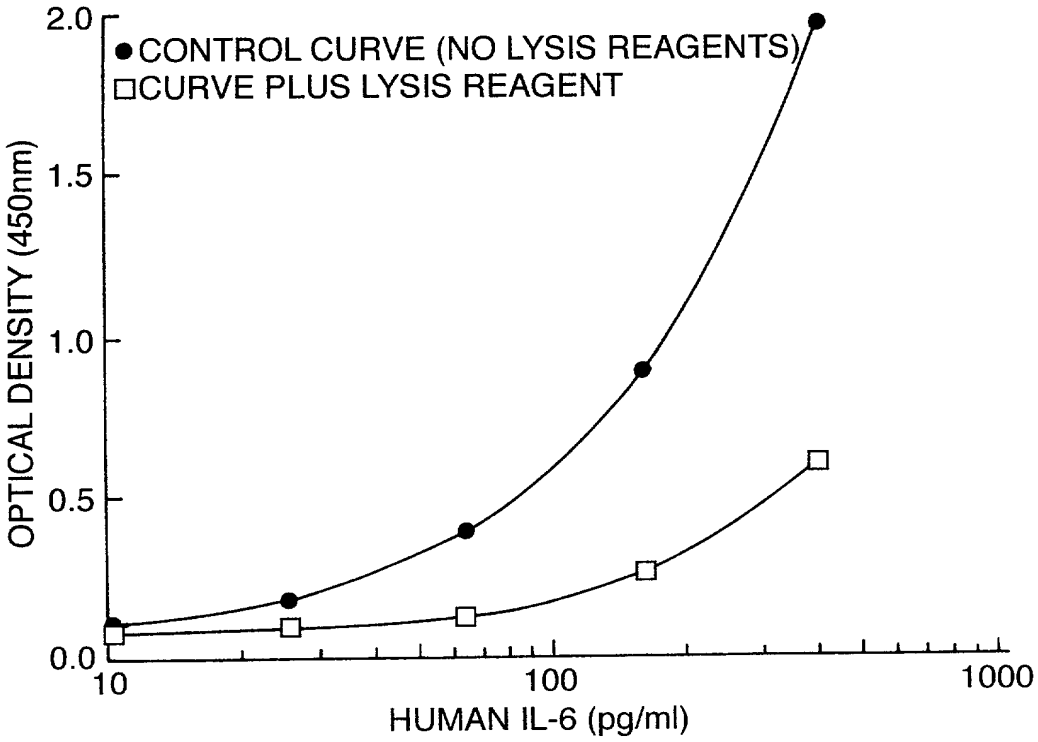

2.0
1.5
1.0
0.5
0.0
OPTICAL DENSITY (450nm)
● CONTROL CURVE (NO LYSIS REAGENTS)
□ CURVE PLUS LYSIS REAGENT
10
100
1000
HUMAN IL-6 (pg/ml)

Fig.6.

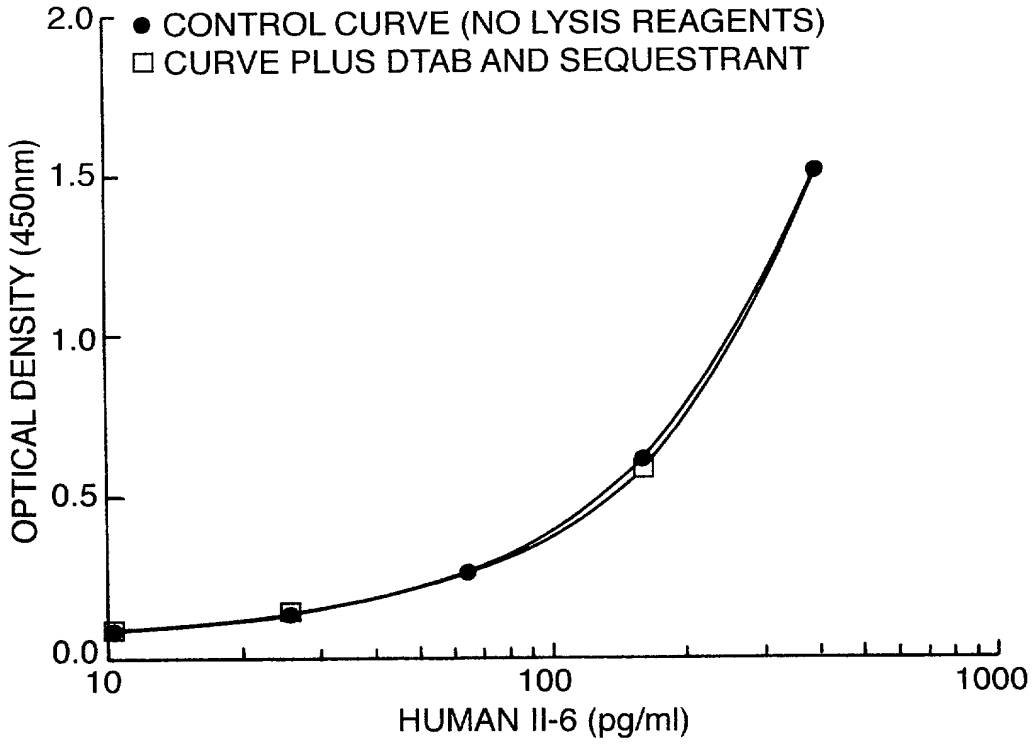

2.0
1.5
1.0
0.5
0.0
OPTICAL DENSITY (450nm)
● CONTROL CURVE (NO LYSIS REAGENTS)
□ CURVE PLUS DTAB AND SEQUESTRANT
10
100
1000
HUMAN Il-6 (pg/ml)

Fig.7.

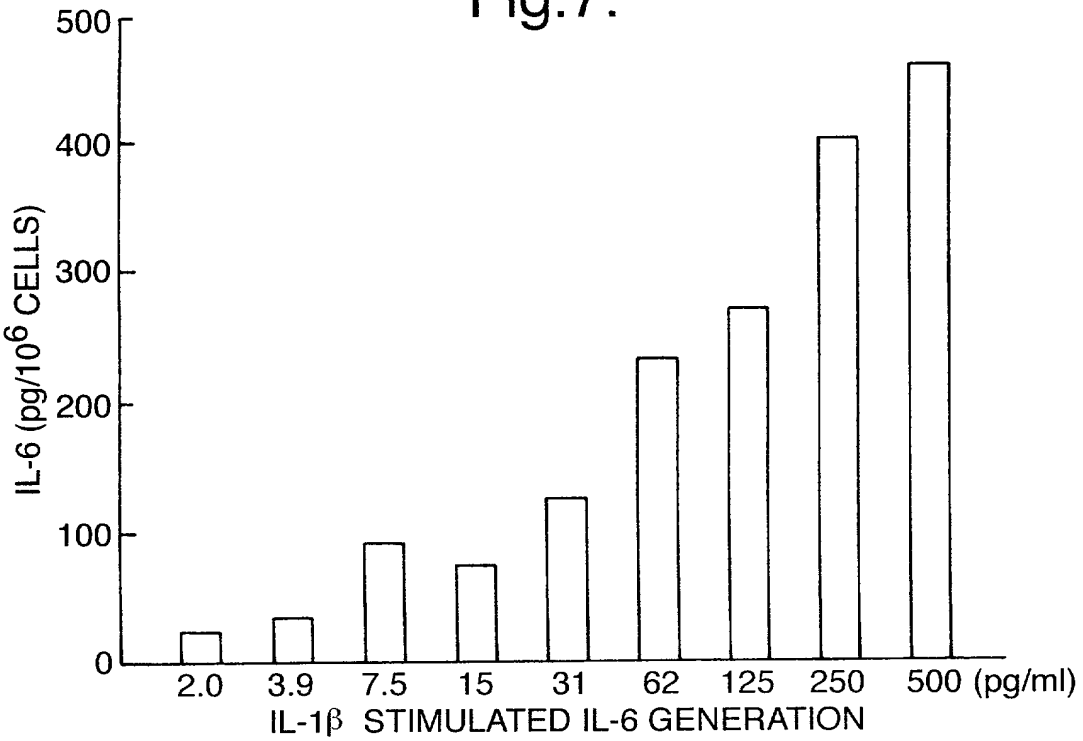

500
400
300
200
100
0
IL-6 (pg/10^6 CELLS)
2.0
3.9
7.5
15
31
62
125
250
500 (pg/ml)
IL-1β STIMULATED IL-6 GENERATION

Fig.8.

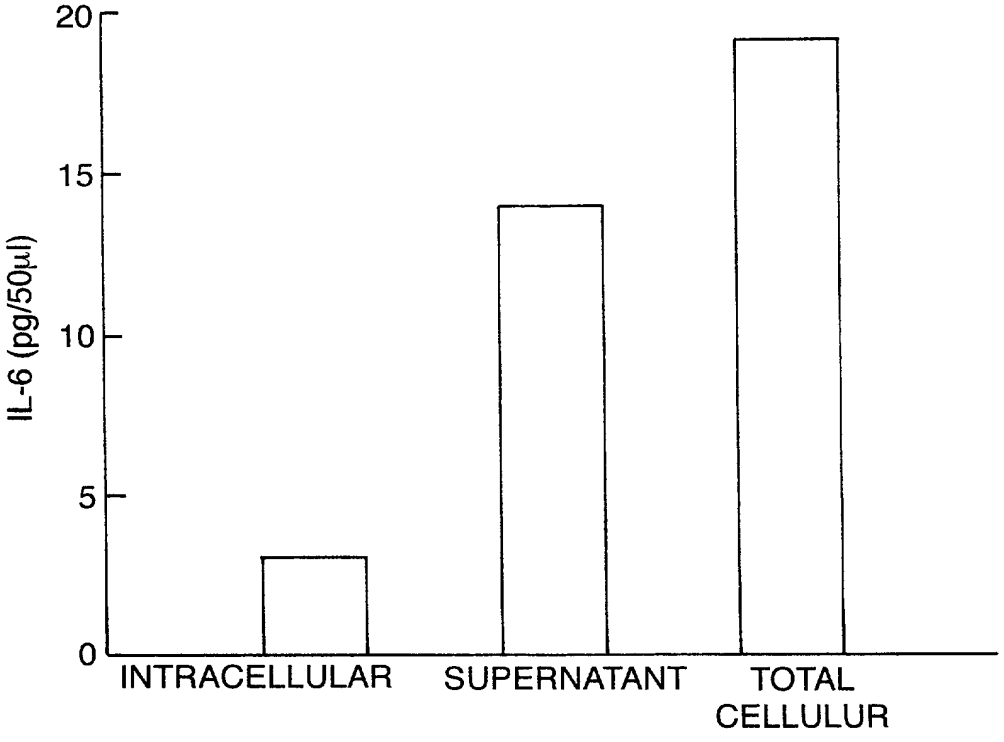

20
15
10
5
0
IL-6 (pg/50μl)
INTRACELLULAR
SUPERNATANT
TOTAL CELLULUR
IL-1β STIMULATED IL-6 GENERATION

Fig.9.

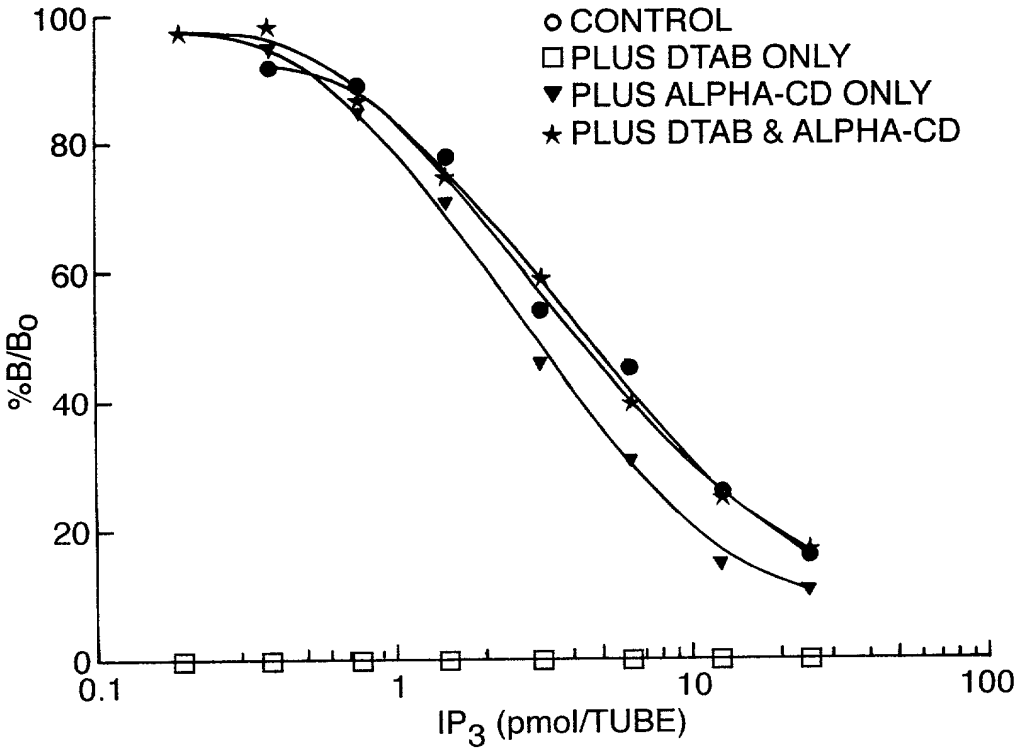
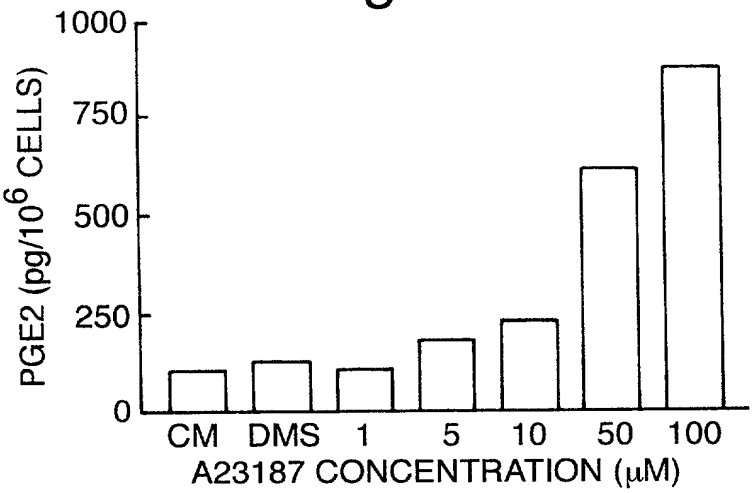


Fig.10.



CM= CULTURE MEDIA ONLY (ZERO A23187)
DMS= CULTURE MEDIA PLUS DMSO (ZERO A23187)

Atty. Docket No: _____

DECLARATION FOR PATENT APPLICATION

As a below named inventor, I hereby declare that my residence, post office address and citizenship are as stated below next to my name. I believe that I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled "IN SITU CELL EXTRACTION AND ASSAY METHOD," the specification of which (check one): ☒ is attached hereto: ☐ was filed on _____ as Application Serial No. _____ and was amended on _____ (if applicable); ☐ was filed as PCT International Application No. _____ on _____ and was amended under Article 19 on _____ (if applicable). I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment(s) referred to above. I acknowledge the duty to disclose to the Patent and Trademark Office all information known to me to be material to patentability as defined in Title 37, Code of Federal Regulations, §1.56. I hereby claim foreign priority benefits under Title 35, United States Code, §119 of any foreign application(s) for patent or inventor's certificate or of any PCT international application(s) designating at least one country other than the United States of America listed below and have also identified below any foreign application(s) for patent or inventor's certificate or any PCT international application(s) designating at least one country other than the United States of America filed by me on the same subject matter having a filing date before that of the application(s) of which priority is claimed.

			Priority Claimed	
97301398.0	EP	3 March 1997	☒	☐
(Number)	(Country)	(Day/Month/Year Filed)	Yes	No
9715704.4	GB	24 July 1997	☒	☐
(Number)	(Country)	(Day/Month/Year Filed)	Yes	No

I hereby claim the benefit under Title 35, United States Code, §120 of any United States application(s) or PCT international plication(s) designating the United States of America listed below and, insofar as the subject matter of each of the claims of this plication is not disclosed in the prior application(s) in the manner provided by the first paragraph of Title 35, United States Code, 12, I acknowledge the duty to disclose to the Office all information known to me to be material to patentability as defined in Title ', Code of Federal Regulations, §1.56 which occurred between the filing date of the prior application(s) and the national or PCT ternational filing date of this application:

pplication Serial No.)	(Filing Date)	(Status-Patented, Pending or Abandoned)
pplication Serial No.)	(Filing Date)	(Status-Patented, Pending or Abandoned)

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information id belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and e like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that ich willful false statements may jeopardize the validity of the application or any patent issued thereon.

POWER OF ATTORNEY: I (We) hereby appoint as my (our) attorneys, with full powers of substitution and revocation, prosecute this application and transact all business in the Patent and Trademark Office connected therewith:

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State or Country	**State or Country**
United Kingdom	United Kingdom
Date ☒ 6 February 1998	**Signature** ☒ JK Horton

☐ See second page for additional inventor(s)

See reverse for relevant rules & statutes